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CONTENTS

GROWTH REQUIREMENTS OF BACTERIAL VIRUSES[1]

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The problem of the growth requirements of bacteria is generally considered to consist of a description of those organic and inorganic components in the medium which must penetrate the organism to become available for various metabolic or structural rôles essential to the production of a like organism[2]. It also includes a definition of these rôles, metabolic, structural, or both. Implicit in an approach to the problem is the knowledge that the bacteria contain the enzymes capable of synthesizing and assembling their own macromolecular components. Since the growth of bacterial viruses has to date been shown to occur only in an ordered enzymatic environment, as in living cells[3], the problem under discussion must be expanded to include the question, "How much of the enzymatic functions of virus synthesis are accomplished by the host cell?" or, conversely, "To what extent does the virus contribute to its own synthesis?" Therefore, it is clear that the problem of the growth requirements of viruses is a far more complex problem than for bacteria.

The environment essential for multiplication of any virus consists of the numerous organized macromolecular components of a living cell. These components, in turn, may be affected by variations in the external environment of the cell. Since it is difficult to vary the interior of the cell directly and controllably, the external environment of the infected host has been varied most frequently

[1] The words "growth" and "multiplication" must be clearly distinguished in discussing the development of bacteria. In most studies of bacterial requirements, conditions have been sought which are satisfactory with respect to both increase in protoplasm (growth) and increase in the numbers of organisms (multiplication), although under special conditions, as under the influence of penicillin or ultraviolet irradiation the two phenomena are separable. In discussing bacterial viruses, however, we will use "growth" and "multiplication" interchangeably, since no evidence has yet appeared suggesting that growth and multiplication are two distinct, separable processes in these organisms, i.e., that one intact bacteriophage particle may augment in matter, maintaining its integrity as an organism (growth) prior to the transformation of this matter into more than one bacteriophage particle (multiplication). In constrast to the situation for bacteria, studies of distribution of size of the viruses suggest that this mechanism does not exist within that group (66) (however, see electron micrographs of agent of feline pneumonitis (35)).

[2] This statement of the problem has developed primarily from attempts to grow large numbers of various microörganisms in liquid media under defined conditions. It may be assumed that the nature of the problem will be expanded to that of a more complete statement of an organism's ecological niche when such problems as the speciation and evolution of bacteria, etc., are considered.

[3] The recent report of bacteriophage increase in cell-free filtrates of lysozyme-disrupted staphylococci (42), can not be considered an adequate proof of the production of bacteriophage in cell-free media. It will be necessary for Krueger, *et al.* to demonstrate increase of phage titer after large numbers of transfers in these media, to eliminate the possibility of disaggregation effects.

in an approach to the effect of environmental changes on virus growth. The interpretation of any change in virus growth as a result of adding some factor to the medium is then invariably subject to the following types of question: (*a*) Does the substance go to the formation of virus, host, or both? (*b*) Is the substance affecting the functioning of the enzymes of the virus, host, or both? And so forth. It should therefore be clear that the interpretation of any observed change in virus growth consequent upon some change in the environment of the host depends on a great deal of detailed knowledge of the mechanism of virus growth and of the metabolism of the infected cell.

It will be necessary, therefore, to proceed to a consideration of the data on phage multiplication and the metabolism of infected bacteria as a basis for a discussion of the effects of various environmental changes on virus growth.

Since the early work of D'Herelle (26), it was known that a reproductive cycle for bacteriophage consists in the adsorption of the phage to its host bacterium, multiplication of the virus within the infected cell and, finally, lysis of the infected cell, liberating the enclosed multiplied virus into the medium. The rigorous experiments of Delbrück and his collaborators summarized in various reviews (18, 19) have confirmed the three main features of the cycle and have provided methods for the quantitation of these phenomena. These simple and relatively precise methods have permitted careful handling and examination of infected cells, which must be the focus of any analysis of virus multiplication, at least for the present. This obvious fact has by no means been a matter of common acceptance in all phage studies, and indeed, has rarely been considered in studies of the metabolism of animal or plant tissue infected with virus.

These methods may be described briefly. The assay of host cells (bacteria) is usually accomplished by an estimate of cells capable of forming colonies. In a few studies, direct cell counts have been made. The assay of virus makes use of plaque counting techniques (34, 37) which measure the ability of a single phage particle to multiply in the presence of a large excess of growing bacteria fixed in a thin, continuous film on an agar plate. An older, unreliable technique of virus assay made use of the time of final lysis, after addition of a suspension of phage to a large excess of growing bacteria under specified conditions. This procedure involving a series of phage reproductive cycles has been abandoned largely for the plaque counting technique, although it is still used occasionally in the design of experiments, the adequacy of which will be considered below.

Bacteria may be controllably infected by making use of the fact that the adsorption rate is a function, among other variables, of the product of the concentrations of bacteria and phage (20). Rapid adsorption is accomplished at relatively high titers of the reactants in media of suitable ionic strength and is stopped by dilution. The numbers of infectious centers are estimated by determining the total number of infectious centers in the suspension and subtracting the number of infectious centers remaining (free unadsorbed virus) after removal of infected cells by centrifugation (21). Or, free virus may be eliminated by treatment with antiviral serum which still permits virus growth within infected cells. The numbers of infected cells after mixture of known amounts of bacteria and virus may be determined simply by use of the Poisson distribution.

The average multiplicity of infection, i.e., the ratio of virus particles per bacterium, may be established controllably and determined readily by making use of these methods. The study of metabolic phenomena in the infected cell by direct estimation of chemical changes has depended on the ability to ensure that all the cells of a given suspension were indeed infected (9, 10) or, conversely, on the course and quantitation of virus liberation from infected cells by the one-step growth technique (18, 11–13, 33). Direct microscopic examination and the change in turbidity (27), have been used to study lysis. An experimental procedure which follows a single cycle of virus multiplication within infected cells by the use of these principles and techniques may be called a one-step growth experiment. Some workers who have presented the most unsatisfactory experiments and unwarranted conclusions have criticized the one-step growth experiment as too limited.

Much of the data to be presented are derived from the study of a set of seven T viruses, reviewed by Delbrück (18), infecting a strain of *Escherichia coli*. Some of their genetic variants, notably the r mutants of T2, T4, and T6 have been described by Hershey (38, 39) and Doermann (27). The even-numbered phages T2, T4, and T6, which have been used most, are large tad-pole shaped particles which contain large amounts of desoxyribose nucleic acid (DNA) in addition to protein. Some chemical and physical properties of these phages have been described in detail (9, 10, 40, 61, 67, 73). T3 and T7 are small, spherical phages (18).

ADSORPTION AND PENETRATION

Although we are concerned in the main with the second part of the cycle, i.e., multiplication within the infected cell, it is not clear to what extent adsorption or lysis may be dissociated from the multiplication phenomenon. For instance, although phage will adsorb to dead bacteria incapable of phage synthesis, it is not known whether the adsorption to live cells initiates a set of host reactions essential to virus synthesis, or constitutes, in itself, a penetration mechanism, or perhaps both of these, or whether adsorption is only comparable to the landing of a troop carrier which must then unload its assault equipment in the form of a penetration mechanism. Although certain evidence suggests the possibility of a penetration mechanism for bacterial viruses, the data are fragmentary and clear interpretation is not yet possible. Anderson has observed that T2 phage disrupted by ultraviolet irradiation will hasten the lysis of heavily irradiated *E. coli*, which are in the main already autolysing (3). Although this has been interpreted as being due to a lysing enzyme derived from phage (56), the effects may have been provoked by some trigger mechanism of phage ghosts or extract which set off host enzymes. Tests of intact or disrupted T2 phage for depolymerases such as proteinase, desoxyribonuclease, or lysozyme have been negative (14).

It has been shown that many phages require adsorption cofactors, such as tryptophan (4) or isoleucine (5). Recently, it has been reported that one T4 strain requires both tryptophan and Ca^{++} for adsorption (23).

Adams (1) has shown that Ca^{++} is essential for T5 multiplication in *E. coli*,

although not necessary for the multiplication of the bacteria. He has found that T5 will adsorb in the absence of Ca^{++} and that the normal latent period of multiplication within the host is obtained after the addition of Ca^{++}, even though the delay may have been as much as 30 minutes prior to its addition. Thus the need for Ca ions within the first minute after infection may indicate a rôle in an essential penetration mechanism.

Similarly, Wahl has shown that Ca^{++} is essential for the multiplication of several bacteriophages C38 and S13, but not of their bacterial hosts (75). These were not adsorption cofactor effects.

PHYSIOLOGICAL STATE OF THE HOST CELLS

Bacteria in their lag phase will generally produce less virus than will organisms during their exponential phase of growth (however, see (69)). Price has recently described this in staphylococcal systems (57). He has also observed that the incubation of lag phase cells for one hour with a yeast fraction of undetermined nature considerably increased the ability of these cells to produce phage. Since he has found that the addition of this material to cells taken in their exponential phase has little if any effect on the number of virus particles produced from these cells, it is possible that he has found something capable of hastening the conversion from lag phase to exponential phase and not a substance which directly stimulated phage growth. In view of the inadequacy of the data concerning the nature of the differences between bacteria in their lag phase or exponential phase, this type of information does not greatly contribute to clarity.

Since certain bacterial mutations from virus sensitivity to virus resistance appear to result in a loss of synthetic powers for factors essential for bacterial growth, e.g., the mutant resistant to T1, B/1, is generally tryptophan-requiring (2), a wide survey of the nutritional requirements of resistant mutants may yield some clues as to the nature of resistance and steps concerned with virus growth.

For more than a decade attempts were made to establish a relation between virus growth and the multiplication of the bacteria prior to infection. In typical studies some workers found that the rate of virus increase in an infected culture appeared to be a function of the rate of bacterial increase (55). Krueger and Scribner eventually concluded that multiplying bacteria were not essential to virus growth since bacterial growth and virus growth appeared to be separate functions under certain conditions (44). The question has been answered more clearly by the findings that *E. coli* rendered incapable of multiplying by ultraviolet, or X-irradiation, or mustard gas still support virus multiplication (6, 65, 36). It is reported that *E. coli* suspended in a phosphate buffer supplemented by glycine or derivatives can support virus growth although unable to multiply (69). This problem is relevant only in the most indirect fashion, since it adds the whole problem of the nature of cell division to that of phage growth. As will be seen in the next section, cells infected with T2 and other phages, are incapable of multiplication (9).

Many workers have studied the effect of penicillin on bacterial division and phage growth (53, 54, 64, 58, 43, 29). Although it is agreed that penicillin-

inhibited bacteria can still support phage growth, it is clear from the more detailed studies of Elford that pretreatment of the bacteria with penicillin for some minutes is deleterious to mechanisms for phage synthesis, primarily affecting virus yield but not the duration of the latent period. The pattern of effects is somewhat variable for each bacterial species. Cytological details of staphylococcal phage growth in the presence of penicillin have recently been observed in the ultraviolet and electron microscopes (68).

A survey of the action of various inhibitors on bacterial growth, phage activity, and the ability of the infected cell to support virus growth shows that all possible types of associated and dissociated effects have been obtained. This is presented in table 1. Thus, in group 1, compounds affect cell and virus separately, as well as the infected cells; in group 2, effects are noted on the cell alone

TABLE 1

The activities of various inhibitors studied in phage synthesis

GROUP	INHIBITOR[4]	REFERENCE	INHIBITION OF BACTERIAL MULTIPLICATION	DIRECT INACTIVATION OF PHAGE	INHIBITION OF PHAGE GROWTH IN INFECTED CELLS
1	Streptomycin	40, 15	+	+	+
2	5-Methyl tryptophan	11, 12	+	0	+
2	Methionine sulfoxide	33	+	0	+
2	Sulfathiazole	24	+	0	+
2	Cyanide, iodoacetate	70, 60	+	0	+
3	Temperature 45°	46	±	0	+
3	Citrate	72, 8, 7, 1	0	0	+
3	Proflavine	31, 32	0	0	+
4	Penicillin	53, 54, 64, 58, 43, 29, 68	+	0	0
5	Indole	23	0	+	0
5	Antiphage sera	22	0	+	0

The signs +, 0, and ± indicate the existence of, absence of, and partial inhibition, respectively.

and on virus growth but not on the phage alone; in group 3, virus growth is affected but not the host alone; in group 4, effects are observed on the cell alone without affecting virus growth; and, lastly, in group 5, we find effects on the virus alone without affecting host cell or virus growth. In a later section, we will attempt to correlate these results with the particular stages of the phage reproductive cycle.

VIRAL INHIBITION OF CELL MULTIPLICATION

When *E. coli* B is multiply infected with a T2 or T4 strain of phage in a synthetic medium, the opacity of the culture remains constant until lysis begins

[4] It would be desirable to be able to consider the recently reported effects of pectin on T2 multiplication in B (50). The data and the experiments themselves are inadequate to permit conclusions at this time. It is hoped that adsorption data and one-step growth curves under the influence of pectin will be made available shortly as well as other experiments.

(9). In nephelometric studies with T2, T4, or T6 strains, the light scattering of a suspension of infected bacteria does not exceed the value obtained immediately prior to addition of virus (27). In contrast, the turbidity of cultures multiply infected with T1, T3, and T7 continues to increase until lysis (74); the significance of this observation is not clear.

The cell division of infected bacteria has never been observed. Cytochemical studies by various workers have shown a rapid transformation of the nuclear apparatus of infected *E. coli*. The conclusion that infected bacteria do not multiply also may be inferred from the numerous observations of the constancy of the number of infectious centers, as determined in one-step growth curves during the latent period of virus multiplication in multiply infected bacteria. Ultraviolet irradiated T2, T4, and T5, which are unable to multiply under conditions of single infection, also inhibit bacterial multiplication (47, 9). This fact may be used in the assay of that type of inactivated virus.

This type of data has scarcely been approached in other plant and animal virus studies. It is possible that virus-infected cells do indeed multiply. Two borderline cases of cytoplasmic inheritance, effected through germ cells, e.g., kappa in Paramecia strains, and the CO_2-sensitivity factor in Drosophila, would be instances of this, if it were considered that the inherited factors were indeed viruses. Also, it is possible that, as reported in the phenomenon of lysogenicity, bacteria may multiply while carrying virus (however, see (18)). Nevertheless, no evidence has ever been presented that phage multiplication occurs in a bacterium which is itself multiplying at the same time. These problems are clearly pertinent to the problem of the nature of tumor viruses, latent viruses, etc.

SYNTHESIS AND ENERGY SUPPLY

The cessation of multiplication of the infected cell implies a deep-seated disturbance of the metabolism of the host cell. When the oxygen consumption of two aliquots of a normal culture of *E. coli* are compared in the presence or absence of virus T2 or T4 in a lactate-containing synthetic medium, it is observed that in the infected culture the normal increase of rate is prevented, concomitant with the inhibition in multiplication of the cells. Simultaneously it may be noted also that the rate of O_2 consumption and the respiratory quotient in the infected culture is the same until lysis, as the rate found for that culture immediately before infection (9). A similar observation has also been made for staphylococci infected by phage in broth (63). It may be concluded from these studies that while the original respiratory enzymes are unaffected by infection, the synthesis of bacterial factors (presumably these enzymes) essential for increased oxidation is totally inhibited.

In confirmation of this inhibition of enzymatic increase under conditions of infection, Monod and Wollman (51) have demonstrated that strains of *E. coli* infected by virus B_2 are unable to synthesize adaptive enzymes for the utilization of lactose. Thus cells unadapted to lactose were unable to synthesize virus with lactose as the sole carbon source. Cells in which adaptive enzymes were syn-

thesized before infection, maintained their respiration in the presence of this substrate after infection, and were able to support virus reproduction with lactose as the sole carbon source.

In these cases it may also be concluded that, inasmuch as the overall energy supply, as determined by the rate of oxygen consumption and the R.Q., remained the same in the infected cell performing phage synthesis as in the uninfected cell, the energy for phage synthesis is primarily a product of host enzyme activity shunted from use in bacterial synthesis. Since, as will be described later, almost all, if not all the products of infected cells are phage components, the energy for phage synthesis is the major element of the total energy supply in the total respiration measured. That this energy supply is essential for phage synthesis is shown in the following experiments. When the oxidative respiration of strains of *E. coli* is inhibited by depleting the medium of oxygen with a stream of nitrogen, or by the addition of cyanide or iodoacetate to the medium, infection with T2r$^+$ induces lysis from the moment of infection, as shown in figure 1 (14). Phage is not synthesized in this system and the infecting virus is lost. It appears that one virus particle can initiate this lysis-from-without[5] in the absence of a suitable energy source, and very active proteinase and desoxyribonuclease not observed in the virus have been demonstrated in these lysates. However, when adequately respiring cells are infected and subsequently treated with nitrogen or cyanide, the tendency to lyse is somewhat reduced. It would appear that infection with T2 produces a rapid reorganization of cell substance, which in the absence of the host's energy supply is uncontrollable, leading to autolysis and disrupting of the structures essential to phage synthesis. The reorganization being completed, the infected cell is less prone to the anarchy induced by cutting off of the energy supply, until, as Doermann has recently shown (28), intact virus particles are present in the cell. This worker has used the technique of lysis-from-without by means of excess phage and respiratory poisons, separately and concomitantly, to demonstrate intracellular phage during the latent period. Similarly, the turbidity of unadapted infected cells in lactose as the sole carbon source was observed by Monod and Wollman (51) to slowly decrease from the beginning of infection, i.e., under conditions where an external energy source was unavailable. It should be noted that lysis of cyanide-treated cells is marked upon the addition of T2r$^+$ and T4r$^+$, but does not occur with T7 (14) and T4r (28).

Price has shown that cyanide, iodoacetate, and azide inhibit phage synthesis in penicillin-treated staphylococci capable of supporting phage growth, and that these agents lower the ATP content of the cells (60).

In addition to the very early reorganization of cell structure and metabolism just described, it is considered probable that some types of infecting virus par-

[5] Delbrück has coined the term "lysis-from-without" to describe lysis without multiplication caused by infection with large amounts of phage, *ca.* 100 particles per cell (25). It is probable that this type of induction of lysis is of the same autolytic type as chemical induction of lysis in singly-infected cells. The phenomenon is clearly independent of the adsorption capacity of the bacterium.

ticles, i.e., T2, T4, and T6, are disrupted within the cell and that the numerous separate fragments provide the models for the duplication process. An infecting virus particle has never been recovered. Doermann's study of intracellular virus has shown that from infection to about one-half the duration of the latent period, when intact particles first appear, the average intracellular virus content per cell is far less than 1. The hypothesis of the early disaggregation of the hereditary factors of T2, T4, T5, and T6, prior to recombination in newly synthesized virus, has been discussed by Luria (47).

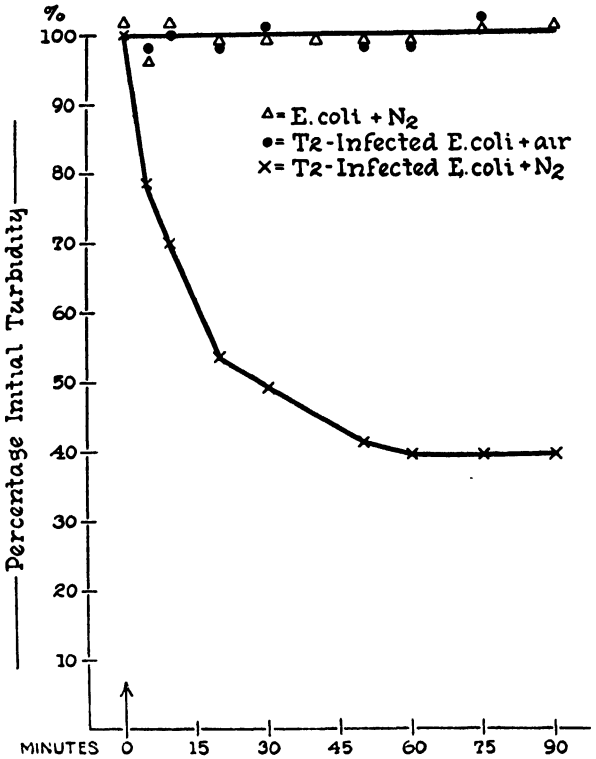

FIG. 1. Turbidimetric study of *E. coli* strain B infected aerobically and anaerobically. Organisms in their exponential phase of growth were centrifuged and resuspended in a lactate medium (9) at 8×10^9 cells per ml. Air or N_2 was passed through the medium for 5 minutes before addition of a purified preparation of $T2r^+$ to give a virus to cell ratio of 2.7.

ASSIMILATORY PROCESSES

Although it has been noted that infected cells are unable to produce more bacteria and certain types of bacterial enzymes, we have found that synthesis proceeds in these cells at a very considerable rate (10, 16). This has been studied in the main in $T2r^+$ and $T4r^+$ systems under conditions of multiple infection, although a beginning has been made in r phage systems. The basic characteristics of the synthetic processes are presented in figure 2.

It may be noted that protein is synthesized from the beginning of infection,

while under the conditions of this experiment, the increment in desoxyribose nucleic acid (DNA) begins about 7 minutes later. The rate of synthesis of this DNA in infected cells is about 4 times that in normal cells. It has been found that there is little, if any, increase of other protein-bound carbohydrate, and no appreciable increase of ribose nucleic acid (RNA), although normal cells produced

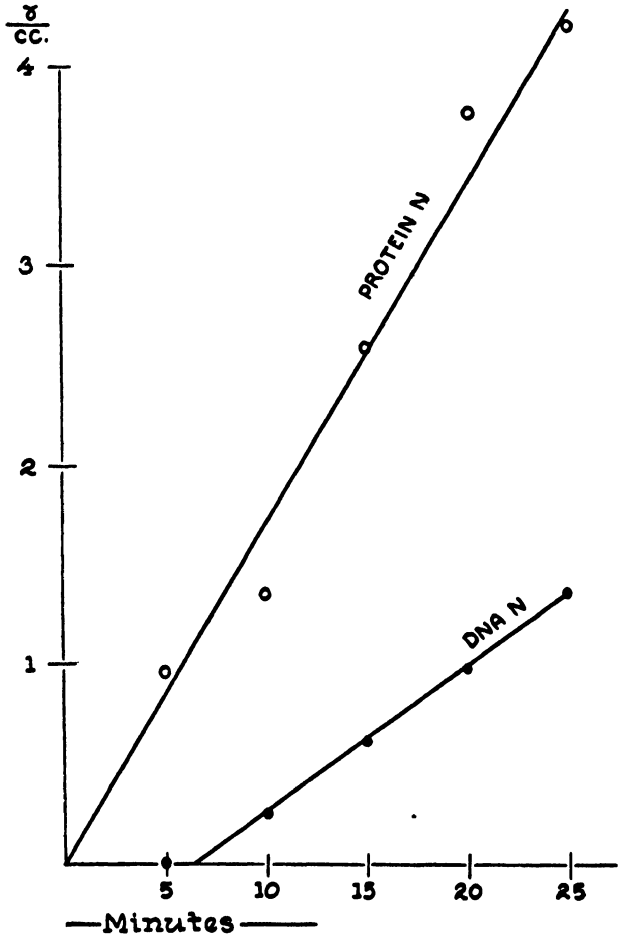

FIG. 2. DNA and protein synthesis in *E. coli* strain B infected with T2r+ virus before the onset of lysis inhibition. The ordinate presents increments over initial values. For experimental details, see (16).

about 3 times as much RNA as DNA. Indeed the RNA of infected cells is essentially inert, as determined by turnover studies with radioactive P^{32}.

The phosphorus of the virus DNA has been shown to come, in largest part (*ca.* 80%), from the medium after infection (17). The origin of the remainder of the P is not known. Similar results with T6r+ have recently been reported by Putnam (62). That the DNA synthesized is actually virus DNA is revealed by the fact that more than 70% of the newly synthesized DNA may be isolated

in virus (17). Losses may be expected in isolation experiments due to readsorption in the high concentrations of bacteria necessarily used in these experiments. Correlations of virus equivalents of the newly synthesized DNA, as determined from analyses of isolated virus, and the virus actually found to be produced within those cells, agree within 10%. Finally, it has been observed that all of the protein-bound phosphorus assimilated after infection is found in the DNA and, hence, in the virus fraction, although in normal cells only $\frac{1}{4}$ to $\frac{1}{5}$ of the assimilated phosphorus becomes DNA.

Price has estimated the nucleic acid content of an infected culture of penicillin-inhibited staphylococci (60). He found that the DNA and RNA contents of infected and normal cultures were identical and concluded that phage multiplication does not involve a disturbance of nucleic acid metabolism. Price's conclusions are unwarranted because he infected large numbers of organisms with a small amount of phage and harvested his infected culture "15 to 30 minutes" before lysis ensued. Under these conditions, either only a few per cent of the bacteria were indeed infected or, when harvested, they had been infected for too short a period to reveal significant differences in nucleic acid metabolism.

It has been observed that, in media which do not contain phosphorus, a very small amount of T2 synthesis in *E. coli* is nonetheless possible, in contrast to the impossibility of such synthesis in media without an added carbon or nitrogen source (33). In related studies, it was found that adenine and the nucleosides guanosine and desoxyguanosine may stimulate virus synthesis in synthetic media (33, 13). Guanine, uracil, thymine, and cytosine had no detectable effect in this system. In view of the importance of nucleotides as coenzymes in energy supply, it cannot be inferred that the stimulatory effect of the nucleic acid derivatives were necessarily due to their direct incorporation into virus.

Somewhat less is known about protein synthesis in infected cells than about nucleic acid synthesis. Considerably more protein is synthesized in these cells in the latent period than can be accounted for by the numbers of virus particles produced. However, under the special conditions of lysis inhibition with r^+ strains (27), protein synthesis is inhibited after the normal latent period is completed, and the numbers of virus particles liberated account at the moment of final lysis for the protein produced in infected cells. Indeed this balance is attained after the initial excess of protein, produced before lysis-inhibition begins, has been depleted and this implies that that excess was virus protein (10, 16). In the case of the turnip yellow mosaic virus disease, the presence of both infectious viral nucleoprotein and non-infectious viral protein in large amounts has recently been studied in detail (49).

Since it has been found that some intact phages, e.g., $T2r^+$, do not react with bacterial antisera (37), it may be inferred that virus proteins which have been synthesized do not have bacterial specificity. Presumably, in the case of r^+ virus-infected cells, all protein synthesis is of a new type. Until the present, no turnover studies have been performed to exclude absolutely the transformation of host protein to virus protein and its replacement by new synthesis. If this possibility were true some small degree of virus synthesis, in the absence of ex-

ternal protein-building elements, might be expected. It is found, however, that the absence of external nitrogen for *E. coli*, or tryptophan in a tryptophan-less mutant of this organism, completely excludes virus synthesis (28). However, despite the suggestive character of the data, considerably more data will be necessary to prove rigorously that all protein synthesis in the phage-infected cell represents synthesis of virus components.

Data exist pertinent to the problem of whether this synthesis of viral DNA and protein is carried on by the enzymes of *E. coli* or those of the virus, or both. The amounts of N- and P-containing polymers synthesized in infected *E. coli* are of the same order as that in the uninfected bacteria. It may be assumed, therefore, that this synthesis is being accomplished by a similar quantity of enzymatic equipment as of that organism. Since a T2 particle is of the order of 10^{-15} gm., and a bacterium weighs about 10^{-12} gm., it appears reasonable to conclude that, unless phage contains enzymes about 1000 times more efficient on the average than those of the bacteria, the weight of infecting phage is inadequate to effect the amount of synthesis required to produce phage.

It may also be pointed out that the ratio of N to P appearing in polymers in the T2r$^+$-infected cell before the onset of lysis inhibition is the ratio found in these cells, rather than the distinctive ratio characteristic of T2r$^+$ virus. Finally, we may note that the rate of protein and DNA synthesis in *E. coli* strain B infected with about 5 T2r$^+$ particles is constant. Since the rate of synthesis does not change whether there are five or more than one hundred virus particles in the infected cell, we may infer that the virus is not the enzymatic center of DNA or protein synthesis, and that new centers have not been synthesized or, if synthesized, are not being used for virus synthesis.

It follows from these considerations that the greatest part of the virus, its polymeric structure of nucleic acid and protein, is synthesized by bacterial enzymes according to the bacterial metabolic relations of energy supply and assimilation of nitrogen, carbon and phosphorus. However, they are completed into new types of end products whose specificity is determined by portions of the infecting virus particle. Evidence for the existence of metabolically active enzymes of the bacterial viruses will be discussed in another section.

THE ORGANIZATION OF VIRAL COMPONENTS

The time course of intracellular viral synthesis has been studied by Doermann, making use of the lysis-from-without phenomenon previously described. It has been demonstrated by Doermann and Anderson that the same time course of T3 synthesis is obtained when infected cells are disrupted by the relatively slow lysis-from-without technique or by far more rapid and vigorous sonic waves. Unfortunately the latter technique is inapplicable to the larger viruses T2, T4, T5, and T6, and independent test of the lysis-from-without technique for these viral syntheses is not as yet possible.

Doermann and Cohen have recently compared the rate of DNA synthesis and of intracellular T4r synthesis in a strain of *E. coli* B/r/1 multiply infected with T4r. These results are illustrated in Fig. 3. The major point to be made at

this time is that the deposition of DNA occurs several minutes before the completion of intact virus and it may, therefore, be concluded that the laying down of DNA does not complete this virus, T4r. Experiments of Foster (32) on inhibition of phage growth with proflavin and its reversal on dilution suggest that this inhibitor affects some very late step in the completion of the virus particle.

In the light of these correlations of three major stages of synthesis within the latent period, we may briefly consider some aspects of Latarjet's data (45) on

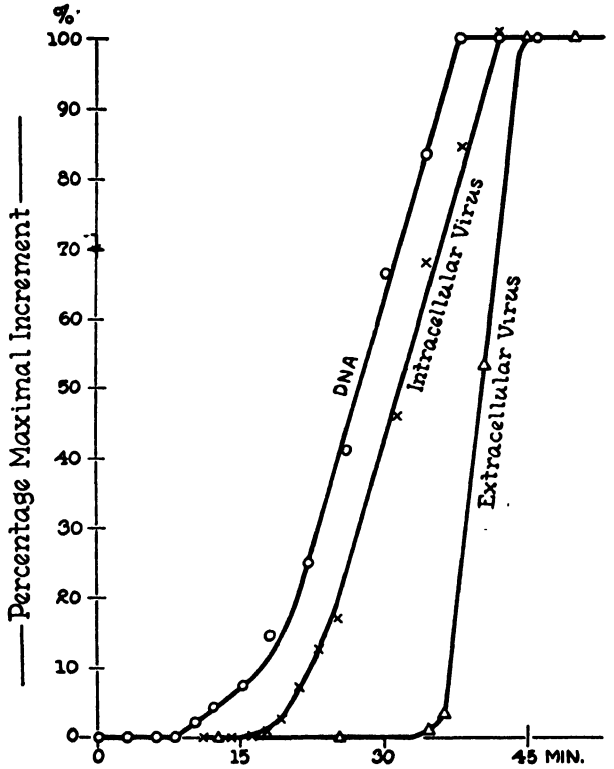

FIG. 3. The synthesis of DNA and intracellular T4r during the latent period of virus multiplication at 30 C within infected *E. coli* B/r/1. The initial virus to cell ratio was $\frac{9.5 \times 10^8 \text{ per ml.}}{1.8 \times 10^8 \text{ per ml.}} = 5.3$. The 100 per cent values per cell in this experiment were 4.0×10^{-14} gm DNA, 115 particles of intracellular phage, and 114 particles of extracellular phage liberated in controls at the end of a normal latent period.

the increase of resistance to X-irradiation of *E. coli* infected by T2r+. He studied the ability of X-irradiated infected cells to liberate virus and found that the shape of the survival curve of singly-infected cells depended on the time in the latent period at which the irradiation took place. Thus, a single-hit type of curve was obtained until about 7 minutes after infection. At 7 minutes, the survival curve of infected cells showed considerable change in X-ray resistance. From 9 minutes to about 13 minutes a multiple-hit curve was obtained, in which multiplicity rapidly increased during this interval. From 13 minutes until the

burst, the multiplicity revealed by this curve remained essentially unchanged but the sensitivity to radiation increased slowly. His data indicate the formation of units, starting at 7 to 9 minutes, which are capable of going on to become complete phage. However, these units are much more resistant (probably smaller) than the complete T2 particle. After 13 minutes, the formation of these units stops, but they then become less resistant (probably larger).

The units of Latarjet cannot be complete T2 virus particles since from Doermann's data the latter would be expected to appear later than 13 minutes in the T2r$^+$ system. Their formation approximately coincides with the beginning of the deposition of DNA and ends when the amount of protein formed is about equivalent to that necessary to complete the virus liberated in the burst. DNA deposition to these units certainly continues and perhaps accounts for some of the decrease of resistance subsequently found in the intact particle. These results are in agreement with prior studies on the ultraviolet irradiation of infected cells (48).

THE PATTERN OF BACTERIAL VIRUS GROWTH

Despite the tentative nature of our present conclusions, and keeping in mind the exceedingly rapid development of the field, we may now proceed to a brief summary of the probable stages of the growth of some phages (T2 type strains).

Virus particles are adsorbed and penetrate within the host cell. The infected cell rapidly reorganizes its nuclear apparatus and its metabolism, although most of its enzymatic activities are unimpaired. These enzymes, derived in the main from the host cell, continue to work on nitrogen, carbon and phosphorus sources, taken largely from the external environment according to the absorption and assimilation patterns of the uninfected cell. However, they no longer build bacterial structure but convert the smaller metabolic building blocks into polymeric substances, DNA and protein, which have the new viral specificities. These new specificities of the products are imposed upon the enzymatic processes presumably by portions of the original infecting virus particle. Protein is synthesized first; the deposition of DNA after 7 to 10 minutes completes the formation of a genetic unit which is not a complete virus particle, possibly lacking the capacity for adsorption and penetration, or some other early stage. The complete particles are formed after the completion of the formation of the genetic units and are liberated by the lysing cell.

DO BACTERIAL VIRUSES CONTAIN METABOLICALLY ACTIVE ENZYMES?

It has been reported that media which were adequate for bacterial multiplication could not support phage multiplication. Thus it has been shown by many workers that Ca^{++} is essential for the multiplication of various phages infecting strains of *E. coli* or Shigella (72, 8, 7, 1, 75), although not necessary for the multiplication of the host. Although, as one interpretation, this would appear to suggest a phage enzyme activated by Ca^{++}, this may not be so, since Ca^{++} may be an essential structural material for these phages.

In coli systems, thiamine was found essential for the multiplication of C36,

which was also Ca^{++}-requiring (75). That thiamine was not an adsorption cofactor was not excluded in these experiments. In staphylococcus systems, nicotinamide had to be added to support phage multiplication (59). Since these compounds can be synthesized by both types of uninfected organism and are active portions of coenzymes probably present in these cells, it may be asked as one of several possibilities if the infected cell has not lost the power of replacement of normally degraded vitamin (see review by McIlwain (52)). In short, this type of evidence, at the present state of our knowledge, may be explained without the hypothesis that the bacteriophage particle introduces into the infected cell new types of enzymes requiring this substance. However, this suggestive evidence clearly points to certain directions of study which may prove or disprove these hypotheses.

NUTRITIONAL REQUIREMENTS FOR VIRUS GROWTH

In the ideal case it would be desirable to demonstrate a rôle for a compound in the virus and its utilization during virus synthesis, as well as the exclusion of a similar rôle for products which may be derived from it. This has been done for tryptophan in $T2r^+$ synthesis. Thus, tryptophanless-*E. coli* B/1 requires tryptophan for T2 multiplication as well as for bacterial multiplication. Tryptophan has been found in $T2r^+$ (9). Although tryptophan may be converted to nicotinic acid in some systems, a nicotinic acid deficiency has been tested for but not observed in T2-infected *E. coli* B (33). In the absence of data suggesting a metabolic rôle of tryptophan other than as one of the building blocks for protein synthesis, and the existence of data indicating that a cell infected by T2 synthesizes only viral proteins, we may conclude that tryptophan is an essential growth requirement for the synthesis of T2 protein.

For the most part T2 has been studied in strains of *E. coli* which can synthesize all of the complex building blocks essential to both bacterial and virus synthesis from simple C, N, and P sources. It has been possible, however, to limit the rate and quantity of virus production in *E. coli* strain B grown in nutrient broth by transferring to media where various protein and other building blocks are eliminated from the medium, as indicated in figure 4. In this type of experiment in which the one-step growth curve of $T2r^+$ has been studied on the broth-grown organisms suspended in broth or the F medium (containing ammonium lactate and inorganic salts) a markedly prolonged latent period and reduced burst size was found in F (33), as contrasted to these functions in broth. Therefore, compounds are contained in broth which make up the difference between the two growth curves.

Bacteria taken from the broth to the F medium did not show a lag period in the F medium but multiplied at a rate about one-fourth of that in broth. As mentioned previously, the elimination of a carbon or nitrogen source from F medium did not permit any virus to be synthesized, while the absence of phosphate or Mg^{++} severely reduced even the small amount of virus production obtained in F medium.

Initially, one-step growth curves were studied on broth-grown organisms trans-

ferred to F media to which single compounds were added (33). Consistent stimulation, reflected in diminished latent period, or increased burst sizes, or both, was obtained with L-phenylalanine, L-aspartic acid, L-proline, L-lysine, L-valine, L-arginine, and L-glutamic acid, the last producing the greatest effect. Guanosine and desoxyguanosine also stimulated virus production.

No single compound of more than 60 common nutritional factors tested reproduced the latent period and burst size observed in broth, although some preparations of indole-3-acetic acid produced marked increments in burst size in

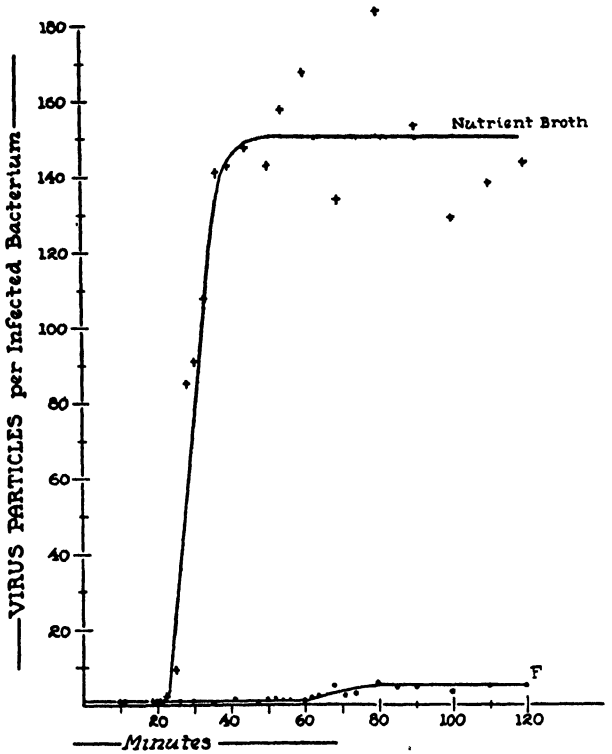

FIG. 4. One-step growth curves for T2r$^+$ in broth-grown bacteria in broth and synthetic media (33). (See text.)

this system. We have not yet been able to establish the nature of this latter effect.

Inhibitory effects were observed in this system with L-leucine, L-serine, and L-cysteine, when these amino acids were added to the minimal medium. The inhibition produced by L-leucine could be counteracted by the simultaneous addition of valine, isoleucine, or norleucine.

It was found that a mixture of amino acids, purines, and pyrimidines added to F medium almost completely restored the latent period and burst size to that observed in broth (33). This observation permitted the use of another group of methods for the study of compounds affecting virus synthesis (13). Since a

complex synthetic medium had been found which gave maximal virus production, it was possible to study the reduction of burst size or increase of latent period when single components were left out of the medium. Also the course of DNA synthesis which appeared to be an approximate measure of virus synthesis could be followed in multiply-infected cells.

It was found that the elimination of some compounds such as tryptophan or leucine from the complete medium only slightly increased the latent period but markedly reduced burst size, as shown in figure 5. In parallel experiments with multiply-infected cells in these same media, it was observed that the time of onset of synthesis of DNA was delayed when these compounds were omitted

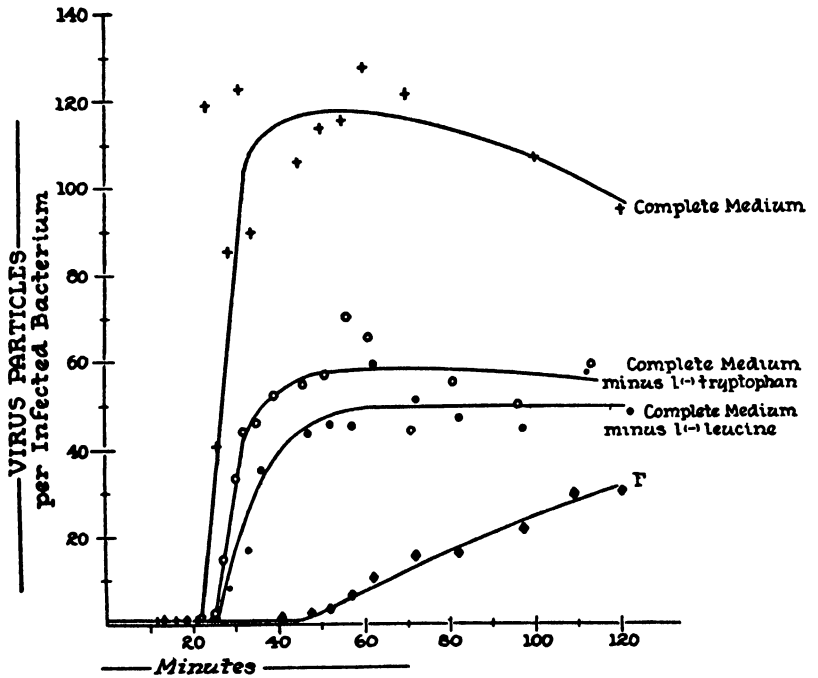

FIG. 5. One-step growth curves for T2r$^+$ in broth-grown bacteria in media of different compositions (13).

but that the rate of synthesis of DNA, once started, was that of the control. In these experiments it was found that L-glutamic acid, L-histidine, L-leucine, L-methionine, L-phenylalanine, L-tryptophan, L-valine, and adenine are compounds which should be present in the medium for maximal production of virus. It should be noted that the supplement and depletion methods yield complementary information.

Spizizen obtained similar results on the importance of amino acids and other substances for the growth of an otherwise uncharacterized *E. coli* phage, P1. He infected in nutrient broth and transferred the sedimented infected cells after more than 7 minutes to various media (69). These media had no carbon source other than the compound added. His data are pertinent in the main to the

multiplication process; they are uninterpretable from the point of view of whether the compound added is used unchanged as a building block for virus synthesis, since the compound provided both the energy and carbon source for the reactions. He lists amino acids and derivatives which on addition to a phosphate buffer can or cannot support virus and bacterial multiplication. He also found that glutamic acid is the most effective single supplement in support of virus growth. The amino acids, alanine, arginine, leucine, serine, isoleucine, cystine, and methionine were unable to support bacterial or virus multiplication. A compound which permitted bacterial increase could in every case support virus growth, while as mentioned previously glycine and glycine derivatives, although inadequate for bacterial increase, permitted virus growth. The experiments of Spizizen on the stimulation of phage growth in glycine anhydride-phosphate buffer by various supplementary compounds are even more difficult to interpret. Marked stimulation is reported for yeast nucleic acid, and various dicarboxylic acids such as oxaloacetate and α-keto-glutarate.

More recently, some preliminary observations have been made in our laboratory on the direct uptake of amino acids by normal and T2-infected *E. coli*. Broth-grown bacteria were transferred to phosphate buffer containing 10 μg of each amino acid, with and without T2 phage. The bacteria were separated from the media at various intervals and the medium was analyzed in unidimensional paper chromatograms, developed in phenol, and sprayed with ninhyrdrin. The utilization of about 7 amino acids by the cells as a function of time could be seen clearly. Whereas the uninfected B strain removed the dicarboxylic acids almost exclusively, it was seen that in addition to the somewhat slower utilization of these acids by infected cells, monoamino monocarboxylic acids and aromatic amino acids were used to a much greater extent (14). More accurate and complete estimations may be presumably made by microbiological assays.

Another approach employed with *E. coli* strains with which depletion techniques cannot readily yield all-or-none effects involved the use of antimetabolites, and other inhibitors, as a clue to metabolic steps important in virus synthesis. Until relatively recently the variety of inhibitors available for study were not of a specific competitive type and the action of various inhibitors on phage growth could not be interpreted readily. Spizizen attempted to use a more specific antimetabolite, aminomethyl sulfonic acid, when he found that amino acids of the external environment, and more particularly, glycine, appeared to be important for virus growth. Unfortunately, although this compound is inhibitory to phage growth, it has been shown by various workers, as well as Spizizen himself, that its action is overcome by various compounds other than glycine (69). In another set of experiments (71) he reports that although succinate stimulates phage growth in glycine anhydride-phosphate mixtures, malonate is not inhibitory.

The available data on inhibition of phage growth by demonstrably competitive antimetabolites are quite sparse. In a very brief report, Delbrück and Luria describe the reversal by PABA of the inhibition of phage growth induced by sulfathiazole (24). Cohen and Fowler (12) have reported a detailed study of the inhibitory action of 5-methyl tryptophan (5MT) on T2 synthesis. 5MT

has been shown to be a specific competitor of tryptophan in protein synthesis (30). Also, some experiments have been made with methionine sulfoxide, which competes specifically with glutamic acid, and results have been obtained similar to those with 5MT (33).

The tryptophan analogue, 5MT, inhibits bacterial multiplication without inhibition of oxygen consumption or change of R.Q., and does not inactivate T2r$^+$ virus or interfere with its adsorption to *E. coli*. At low bacterial concentrations, it will prevent phage growth at low molar concentrations. If the compound is added prior to injection, or at any time up to one-half the latent period, i.e., about 12 minutes in medium F, the cells do not liberate virus in the absence of tryptophan in the medium. Shortly after the end of the latent period, the

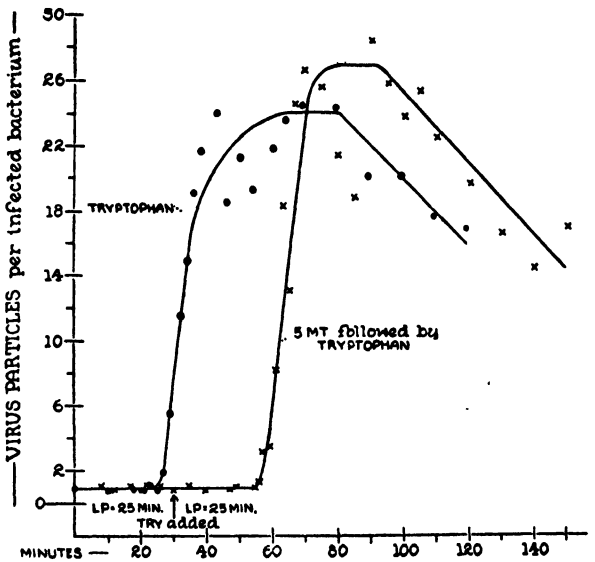

FIG. 6. The inhibition of T2r$^+$ growth in *E. coli* in lactate media by 5-methyl tryptophan (5MT) at infection, and the reversal of the inhibition by tryptophan (12). LP = latent period.

numbers of infectious centers begin to decrease and eventually fall to zero. If the compound is added later than 12 minutes after infection, an amount of virus is liberated which Doermann has recently shown slightly exceeds the numbers of particles present in the infected cell at the time the inhibitor is added (28).

Since the infectious centers can form plaques in the assay method, this means that the multiplication inhibited by 5MT has been reversed, presumably by the tryptophan contained in the broth agar on the plate. It has been shown that small amounts of tryptophan will indeed reverse this inhibition, permitting immediate resumption of phage multiplication. Thus if the addition of 5MT precedes infection and tryptophan is added to the inhibited system after 25 minutes (the normal onset of lysis), a normal latent period of exactly 25 minutes begins and is followed by a normal burst and virus yield, as shown in figure 6. This

signifies that 5MT stops a process requiring tryptophan within the first minute of infection.

If 5MT is used to interrupt the latent period at 12 minutes of a 27-minute latent period and tryptophan is added 15 minutes later, exactly the remainder of the latent period is resumed in normal fashion, as shown in figure 7. Again this indicates a process involving tryptophan at precisely 12 minutes.

This type of study, assuming the availability of sufficiently specific analogues, could readily be extended to many other compounds thought to be important in phage growth. It especially should be applied to the study of various vitamins whose possible rôle in the *E. coli* systems has not yet been demonstrated by any depletion or supplement technique. In addition to its value in revealing the

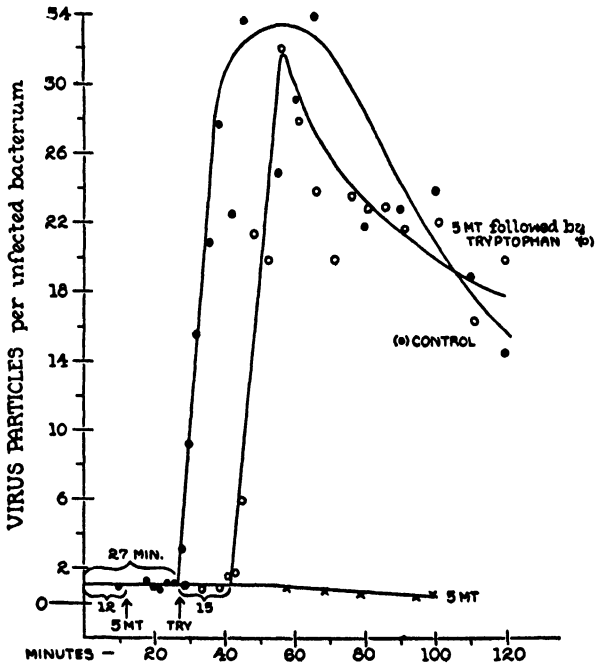

FIG. 7. The resumption of a latent period interrupted by 5MT (12)

existence of tryptophan active during phage growth, the use of 5MT has revealed other phenomena, such as the lysis of inhibited infected cells, regardless of the amount of intracellular virus. Most inhibitors of virus growth, such as 5MT, cyanide, proflavine, appear to cause the lysis of infected but not uninfected cells, and thereby produce the sterilization, with respect to virus, of a population containing normal and infected cells.

SITE OF ACTION OF INHIBITORS OF PHAGE GROWTH

All but one of the inhibiting substances, whose actions were considered in table 1 only in the vague terms of some effect on multiplication, are included in table 2, where an attempt is made to analyze the evidence pertinent to their mode

of action in terms of the more specific knowledge presented on the stages of phage multiplication. This table does not presume to exhaust all the inhibitors known nor the possible modes of action of the various compounds considered. In some instances, there is recorded a probable mode of action as described below. The major purpose of the table is to show the wide variety of sites of action for a compound, metabolite or inhibitor, and that there are methods available to permit analysis as to which of a large number of sites might be involved in the course of affecting phage growth.

Thus streptomycin kills phage and inhibits phage growth at some undetermined site. Tryptophan is an adsorption cofactor for some T4 and T6 strains, and is essential to the synthesis of T2 and T4 protein. Methionine sulfoxide

TABLE 2

Probable mode of action of various compounds on phage growth

GROUP	SUBSTANCE	REFERENCE	SITE OF ACTION							
			Phage	Adsorption	Penetration	Metabolism			Organization	Lysis
						Energy, coenzyme synthesis	Protein synthesis	Nucleic acid synthesis		
1	Streptomycin	41, 15	+	not known						
2	Tryptophan	4, 11, 12, 13, 25	+	+			+	+		
2	Methionine sulfoxide	33				+	+	+		
2	Sulfathiazole	24				+				
2	Cyanide, iodoacetate	70, 60				+				+
3	Citrate	1, 72, 8, 7	+	+	+ ?	not known				+
3	Proflavine	31, 32							+	
5	Indole	23	+	+						
5	Antiphage sera	22	+	+						

Metabolic processes other than energy production, protein, and nucleic acid synthesis are also included; e.g., the process inhibited by sulfathiazole and reversed by PABA.

probably competes with glutamic acid in reactions leading to protein synthesis, formation of intermediates in the tricarboxylic acid cycle, transamination, etc. Sulfathiazole competes with PABA, probably in the synthesis of PABA-containing coenzymes. Cyanide and iodoacetate combine with various enzymes essential to the production of energy from carbohydrate; they also provoke "lysis-from-without". Citrate interferes with Ca^{++} utilization for the adsorption of a T4 strain, the start of multiplication (penetration?) of T5, and lysis of cells infected with various phages. Proflavine appears to affect the completion of T2 and T6. Indole competes with the adsorption cofactor, tryptophan, for T4. Antibody in antiphage sera combines with free phage but does not affect the infected cell.

CONCLUSIONS

In the foregoing, data have been presented on phage multiplication as affected by numerous environmental factors. It has been shown that these environmental factors vary in their complexity, from the ammonium salts which can supply the nitrogen for T2 growth in *E. coli*, to the organized enzymes of *E. coli* which effect synthesis essential to the production of T2. Methods have been devised and described to ascertain more precisely the role of these growth requirements at one or several stages of virus growth. It has been shown, for instance, that tryptophan is a virus constituent and must be provided in the environment to permit the synthesis of virus constituents. This has been demonstrated in tryptophan-requiring bacteria, or by one of the four methods, i.e., the supplement, depletion, antimetabolite or direct utilization techniques in cells capable of synthesizing tryptophan. The interpretation of the results has depended on a great deal of knowledge concerning the growth of a particular bacterial virus and general biochemical information concerning the possible metabolic rôle of chemical compounds. We are at the point where a detailed determination of growth requirements and analysis of their mode of action can be made; with a very few exceptions, such a study has not yet been made. The pertinence of these data and the phage methods to problems of the growth and control of other viruses is so clear as to make one wonder if attempts at their application to these other systems should not be more widespread.

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SOME CONSIDERATIONS OF THE BIOLOGICAL IMPORTANCE OF INTESTINAL MICROÖRGANISMS[1]

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The relationship of intestinal bacteria to the well-being of the host has been of general interest for over sixty years. Despite numerous studies conducted to determine the rôles of these organisms in health and disease, their functions are not completely understood. The kinds of bacteria found in the digestive tract and their distribution have been extensively studied in many animal species including man. It is known that the ecology of the intestines is very complex; changes which have been found to occur in the numbers and kinds of microörganisms in the intestines are difficult to explain.

Nutritionists, bacteriologists and physicians now tend to regard intestinal microörganisms as sources of nutrilites indispensable to the animal host, particularly if the diet is poorly balanced; previously these organisms had been considered only as potential causative agents of gastro-intestinal disturbances. Many of the early investigators considered intestinal bacteria as saprophytes whose putrefactive metabolic products were harmful. Later, it was realized that there are two main types of intestinal bacteria: acid-producing or saccharolytic species, and proteolytic species. The latter group always has been considered harmful or undesirable, but their true significance is not settled. Acid-producing species, on the contrary, always have been deemed desirable for proper conditions in the intestinal tract. Recently, with the help of the rapid advances made in the field of nutrition, considerable evidence has been accumulated which indicates that intestinal bacteria are important in supplying essential growth substances to the host, although it is not certain that they are indispensable. Earlier work now can be re-evaluated with more assurance of drawing valid conclusions. However, there still exists a variety of viewpoints which may eventually be consolidated when more basic work is accomplished.

Probably the first observation of intestinal microörganisms was made by Leeuwenhoek (17) in about the year 1674. Many of the organisms he saw with his simple microscope no doubt were bacteria. Two centuries later, Escherich initiated what is considered to be the first comprehensive study of intestinal bacteria in human infants, and laid the foundation for future work in this field.

THE RÔLES OF INTESTINAL BACTERIA

The present tendency of modern nutritionists to think of intestinal microorganisms in terms of their nutritional importance to their host is based on a

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series of investigations which began with pure speculation by Pasteur. Today it is postulated that intestinal bacteria actually synthesize essential growth factors within the lumen of the gut. In fact, it has been stated by Teply *et al.* (97) "That vitamins are synthesized by intestinal microörganisms has been proved beyond any reasonable doubt."

Following Pasteur's (75) meditations concerning the necessity of microorganisms for life, numerous studies were instituted to shed some light on this highly controversial problem. Metchnikoff (60) was the proponent of the idea that the activities of intestinal bacteria are directly correlated with longevity of the host; a flora consisting mainly of lactic acid bacilli is supposed to subdue proteolytic activities of other microbes, resulting in a more vigorous, longer-lived individual. He had many adherents and some very good evidence to support his beliefs (see Rettger and Cheplin, 81, for a detailed review). Another line of attack, one that is now being pursued more actively than ever, involved attempts to raise various animals with a bacteria-free intestinal tract. It would seem that this type of study should result in a positive conclusion concerning the necessity of intestinal microörganisms for life, or at least, for the general well-being of the host. An exhaustive review of this subject has been presented by Glimstedt (33), hence an extensive discussion is not believed necessary. Nuttall and Thierfelder (71, 72) were the first to raise animals in a sterile environment. They observed impaired growth in some of the guinea pigs raised under aseptic conditions for 13 days. Later, Schottelius (87) and Cohendy (12) succeeded in keeping chicks free from microörganisms for varying lengths of time. Schottelius noted that his bacteria-free chicks grew more slowly and did not survive as long as normal chicks consuming the same sterile ration. Cohendy could find no significant difference between bacteria-free and normal chicks. Thus, the two workers obviously drew different conclusions from their observations. That many of the early failures encountered in raising healthy animals under conditions which would exclude intestinal microörganisms were a result of meager knowledge of nutritional requirements cannot be doubted in view of present-day advances in the nutrition field. Many of the early failures provided excellent evidence for the intestinal synthesis of growth factors. Modern attempts to raise bacteria-free higher animals apparently have been more successful (82, 83), though no conclusive proof of the adequacy of the nutrition of these animals has been established.

The "modern" viewpoint of the rôle of intestinal bacteria is that they provide the host with essential nutrilites. This so-called "modern" viewpoint probably originated with the studies of Osborne and Mendel (73) who attempted to raise rats on highly purified rations containing a single protein, carbohydrate, and fat, plus a salt mixture and agar-agar; these rations now are considered to be highly deficient in essential fractions of the vitamin B complex. They noted the tendency of these rats to become coprophagous, and that this practice alleviated the growth-depressing effects of their various vitamin-deficient diets. When these rats were allowed to choose between their own feces and those from rats on an adequate ration, they invariably chose the latter. Osborne and Mendel believed

that the superiority of feces from normal rats could be attributed to the bacterial flora which, as had been pointed out by Herter and Kendall (41), was markedly influenced by the composition of the diet.

Rettger and Horton (80) and Hull and Rettger (42) coöperated with Osborne and Mendel by performing bacteriological studies on feces from rats fed complete and incomplete rations. A considerable difference was noted between the characteristic flora of rats maintained on the simple "vitamin B" deficient diet and the flora of those on a stock laboratory ration; a simple flora composed of predominately gram-positive bacilli (in some cases nothing but gram-positive bacilli were evident) typified rats ingesting the simple diets of Osborne and Mendel, whereas rats consuming a more adequate ration maintained a more complex fecal flora. They found coliforms to be low in numbers in droppings from rats on either of the two types of diets, but these organisms were particularly scarce in rats on deficient diets. These investigators noted that feeding rats a high milk diet, or a ration high in lactose, had the same effect in simplifying the fecal flora as did feeding the purified, deficient diets of Osborne and Mendel. The gram-positive bacilli found in rat feces were identified as *Lactobacillus acidophilus* and *L. bifidus*.

The earliest suspicions that intestinal synthesis was related to "vitamin B" were stated before this vitamin was shown to be made up of many fractions. Evidence for synthesis was determined by feeding feces of one animal to another animal deficient in "vitamin B." The availability of the synthesized vitamin in the feces to the animal was not considered to any great extent until a number of years later and still remains a controversial issue. Cooper (13) appears to have been the first to suggest actual synthesis of a vitamin by intestinal bacteria. He was able to alleviate polyneuritis in pigeons fed polished rice by feeding them alcoholic extracts of feces of hens and rabbits kept on normal diets. He believed that the anti-neuritic substance was synthesized by intestinal bacteria in the hens and rabbits. This work was repeated subsequently by Portier and Randoin (77) and similar results were obtained. A similar study was made by Theiler and his associates (99) which implicated bacterial synthesis in the rumen of cattle. They believed that a troublesome South African paralytic disease, "lamzietke," might be the result of a nutritional deficiency. In an attempt to reproduce the disease by controlled feeding experiments, they observed that a ration deficient enough in "vitamin B" to produce polyneuritis in pigeons was in every way adequate for cattle. Their assumption was that either cattle required very little "vitamin B," or that intestinal microörganisms synthesized the deficient vitamin, which in turn was utilized by the animal; the latter seemed the more reasonable view. This work opened up the whole new field of rumen synthesis, consideration of which is beyond the scope of this review, although a large share of our knowledge of biosynthesis has been derived from such studies. It is suggested that the reader consult the excellent reviews by Hastings (39), Thaysen (98), Smith (91), and Kon and Porter (47) for further information on rumen synthesis.

Steenbock and co-workers (93) demonstrated more completely the importance

of coprophagy to the rat. They noted that when rats on an apparently normal grain ration were deprived of the opportunity to consume their own feces by the use of raised, wide-meshed screen-bottomed cages, they developed a "vitamin B" deficiency. On this particular ration they calculated that coprophagy supplied over 50 per cent of the "vitamin B" requirement of the rats. Dutcher and Francis (19) confirmed and re-emphasized the findings of Steenbock, *et al.* Heller, *et al.* (40) more directly implicated bacteria as the source of B vitamins furnished in the feces. They observed that rats on a vitamin-deficient diet grew normally if allowed to consume their own feces, and found such feces to contain considerably larger numbers of spore-forming bacteria than feces from rats unable to engage in coprophagy.

In 1927 Fridericia, *et al.* (28) and Roscoe (85) independently published papers which undoubtedly influenced future approaches to nutritional studies. They described spontaneous recoveries of rats which had been depleted on a vitamin B-complex deficient ration. Such recoveries occurred when the rats passed bulky white feces with a high starch content. Fridericia, *et al.* proposed a new term, "refection," to denote this unusual process. It was concluded that the spontaneous recoveries observed in their work were due to consumption of refected feces. The protective phenomenon could be transmitted from animal to animal by feeding feces from the refected animals. Fridericia showed that the agent in the feces was thermolabile and did not pass through a Berkefeld filter. Raw starch was found essential for refection (cooked starch was inactive) although dextrinized corn starch, in certain cases, was found to produce the same effect; sucrose was not beneficial. Fecal contents of refected rats were more acid than those of non-refected rats and showed a different bacterial flora. The hypothesis, therefore, was advanced that "vitamin B"-producing microörganisms had become established in certain animals on a vitamin-deficient diet which had brought about refection. Roscoe emphasized the necessity for control of coprophagy in such nutritional studies in stating: "Whatever construction of cage is used, there are some rats that cannot be prevented from eating their own feces, for they secure them and consume them as they leave the body."

The interesting point concerning these studies on refection was the finding that starch was necessary in the diet for the manifestation of refection. Bliss (8) corroborated these earlier discoveries but was much more successful in producing refection in rats by incorporating raw potato starch in the ration as a substitute for the rice starch used by Fridericia, *et al.* and by Roscoe. Bliss was not too successful in producing refection with either raw rice or corn starches. He demonstrated that the starch was not the source of the B vitamins. Bliss's results showing a differential action of the three different starches may have some relation to the work of Langworthy and Deuel (51), who studied the degree of assimilation of raw corn, wheat and potato starches in three young, healthy men by determining the amount of starch remaining in their stools. Corn and wheat starches were entirely digested, whereas potato starch was found to be from 62.3 to 95.2 per cent digested (average of 78.2). Corn and wheat starches caused no complaints from the subjects, but potato starch caused excessive gas formation

with abdominal cramps. Apparently, the differential effect of various dietary starches has been ignored by nutritionists in attempting to explain the phenomenon of refection. According to Coates *et al.* (11), evidence which appears to explain the cause of refection is accumulating, and seems to indicate that the amount and kind of carbohydrate available to intestinal bacteria influences the degree of vitamin synthesis as well as the qualitative nature of the flora. Fridericia (46) had mentioned this as a problem worthy of more consideration. Investigations elucidating the rôle of carbohydrates in nutrition and their effects upon the intestinal flora will be reviewed in the next section of this report.

Although, as previously pointed out, several of the early nutritionists obtained evidence which was indicative of an elaboration of growth factors by intestinal microörganisms, it remained for Bottomley (9) to observe actual production of a nutritive factor by bacteria. He noted that incubation of peat with a mixed culture of aerobic soil bacteria ("bacterized peat") resulted in stimulation of wheat seedling growth in this peat. This growth-promoting substance could be concentrated by chemical procedures similar to those used in purification of thiamine. Bottomley used wheat seedlings and *Azotobacter chroococcum* for testing his fractions; Najjar and Barrett (68) state that this is "probably the first instance of microbiological vitamin assay." Bottomley's work no doubt gave added encouragement to those who believed or suspected that intestinal microorganisms synthesized essential growth factors which were utilized by the host. However, it was not until the report of Pacini and Russell (74) that evidence was secured showing formation of a substance capable of stimulating animal growth. They showed that a water extract of the typhoid bacillus produced a marked growth response in rats ingesting a "vitamin B"-deficient ration. Similar results were reported subsequently by Bierry and Portier (7) who observed production of animal growth substances by unidentified bacteria which they called "symbiotes."

Many reports of synthesis by bacteria and the needs of bacteria for vitamins required by animals were made in the following years (see reviews 45 and 76) until it became generally accepted that these lower forms of life possessed certain metabolic systems similar to those of higher plants and animals (92). The work of Burkholder and McVeigh (10) and Thompson (100) conclusively pointed to the fact that "obligate" intestinal bacteria are able to synthesize many of the B vitamins required by animals. However, there were indications that certain of these vitamins might not be so readily available to the host because they were retained within the bacterial cells. This effect was also noted by others (1, 65). Also, when one considers the notably poor synthesizing capabilities of certain bacteria, e.g. lactobacilli, enterococci, and clostridia, all commonly encountered in the intestinal tract, it is apparent that a sort of competitive equilibrium must exist within the complex biological system of the digestive tract. An informative study by Benesch (4) resulted in the discovery that the mixed bacterial flora from a human appendix synthesized a considerable amount of nicotinic acid under aerobic conditions, whereas anaerobic incubation brought about destruction of this vitamin. He says, "It seems that normally there exists in the bowel an

ecological system in which aerobic and anaerobic organisms live together, with the result that an equilibrium is constantly struck between the activities of the organisms producing and destroying nicotinic acid."

The prophetic work, which has been mentioned, of Fridericia, *et al.*, and of Roscoe, introduced a new concept in the field of nutrition. Their observations on refection intimated that synthetic factors produced by intestinal bacteria were released in the lumen of the intestine, and that from this source they could be absorbed and utilized by the host. Thus, refection predisposes to absorption of growth factors elaborated by intestinal microörganisms. The initial concept of refection was limited to the rat maintained under specific conditions. It was soon realized that refection was merely a special manifestation of the value of intestinal synthesis to the host. In other words, even without true refection, the host animals might benefit from synthesis of vitamins by intestinal bacteria; this is particularly true in ruminants.

With the discovery of refection, together with the earlier observation of the effects of coprophagy and its value to experimental animals consuming a vitamin-deficient ration, a wealth of indirect evidence rapidly began to accumulate pointing toward the value of intestinal synthesis to animals. The ruminant is unique in that the seat of vitamin synthesis is located in the anterior portion of the digestive tract thereby permitting more efficient absorption of the growth factors synthesized, and thus eliminating the necessity of coprophagy. With non-ruminant animals such as the rat (upon which more nutritional studies have been made than on any other animal), it is a question of the ability of the animal's gut to absorb such factors as may be synthesized before the feces are expelled from the body. Therefore, when an adequate ration is available, intestinal synthesis may act only in a supplementary capacity.

It has been shown by some workers (2, 50, 70) that there is considerable synthesis of vitamin K, riboflavin and biotin in the feces of rats and chickens after expulsion from the body. Recently, Kennard and Chamberlin (44) have reported upon the desirability of allowing floor-litter in chicken houses to accumulate so as to be an available source of some of the vitamins (including the poorly understood animal protein factor). These studies have a further bearing on the rôle of coprophagy in animal nutrition.

A series of studies on the rat by Guerrant and his co-workers (35, 36, 37, 38) has indicated that a large proportion of synthesized riboflavin, thiamine, and the undifferentiated B-complex in general is passed out of the body before these vitamins can be absorbed by the intestinal epithelium. Their results pointed to the cecum and colon as the likely areas for the greatest synthesis. They showed that a cecectomized rat was unable to synthesize sufficient vitamins to grow normally, and required supplements of baker's yeast or feces from rats on an adequate ration. An extremely high yeast population was noted in the cecum, and this led Guerrant, *et al.* to suggest that yeasts were responsible for biosynthesis. Their work also included a study on the differential effect of various carbohydrates upon growth. This subject will be reviewed further in the next section.

Various other studies have been conducted to determine the site of vitamin synthesis and the extent to which the vitamins are available to the host. A

study by Mayhew (58) on the effect of removal of the ceca on the establishment and severity of cecal coccidiosis in chickens showed that uninfected, cecectomized birds on a normal grain ration grew as well as normal, uninfected chicks, except during the first post-operative week, and that removal of the cecum did not in any way impair egg production.

Griffith (34) studied the vitamin "B" and "G" requirements of cecectomized rats and reported only slight differences between his experimental and control animals. Taylor, *et al.* (96) believed that the basal diet Griffith used "may not have been favorable to the maintenance of bacterial growth in the cecum." They found that the cecum of the rat plays a significant rôle in vitamin synthesis only when the animal is maintained on an inadequate diet. They as well as Griffith noted a compensatory effect in their cecectomized rats, however, in that the diameter of the upper colon of operated rats had increased in size. Feces of normal rats fed a sub-optimum "diluted Purina ration" contained more folic acid, pyridoxine, and pantothenic acid but, for no apparent reason, less thiamine. Day, *et al.* (16) produced evidence which indicates that the cecum of the rat may be the main site of vitamin K synthesis as well as for absorption of this fat soluble vitamin. However, their results indicated that the cecum was not the only location for the synthesis of vitamin K since cecectomized rats on a ration deficient only in vitamin K grew well and evidenced no vitamin K deficiency unless one per cent sulfasuxidine was incorporated in the ration; rats with intact ceca on the same ration with sulfasuxidine evidenced no vitamin K deficiency. These results are indicative of the synthesis of vitamin K by bacteria other than those of the coliform group since numerous workers have shown that certain sulfonamides inhibit proliferation of this group of microörganisms (25, 32, 53, 56, 62, 66, 78). Schweigert, *et al.* (88) found that cecectomized rats on complete synthetic rations grew as well as the unoperated controls. Another report by McGregor *et al.* (59), indicates that the sites of biotin synthesis in the rat are in the cecum and large intestine.

That the cecum of the rat is different in certain respects from any other segment of the intestinal tract has been pointed out by Bergeim and colleagues (5, 6). They found the oxidation-reduction potentials to decrease markedly from the anterior to the posterior portions of the gastro-intestinal tract. They obtained E_h values as follows: stomach, +150; upper small intestine, −100; lower small intestine, −100; and cecum, −200 millivolts. Thus, the greatest reducing activity is located within the cecum. They observed an additional interesting effect: lactose, unlike any of the other carbohydrates used, resulted in such a decrease of reducing intensities that positive E_h readings were obtained throughout the intestinal tract for as long as 36 days in rats consuming a lactose ration. This revealing discovery of the anti-reducing effect of milk sugar should provide a stimulus for further research when one considers the effect of lactose on the intestinal flora (81) and the requirement of the young of all mammals for milk. Bergeim and his collaborators also noted that as E_h values became more negative from the stomach to the cecum, pH values of the intestinal contents increased.

No conclusive proof concerning the main locus of vitamin elaboration in all

animals has thus far been advanced. However, several highly enlightening studies have been published which bear mention. Leong (52) reported destruction of vitamin B_1 in the rat, since tissue storage of this nutrilite was extremely low, even when B_1 was used in high concentrations in the ration. Abdel-Salaam and Leong (1) demonstrated synthesis of thiamine by a mixed culture of bacteria from the ceca of rats. They inoculated a thin suspension of cecal contents into broth and after varying lengths of time, the cells and the medium were analyzed for thiamine. They found the most thiamine present in the cells and medium after one day's incubation at 37 C; however, by far the most vitamin was found to remain within the cells, and beyond one day there appeared to be destruction of thiamine. Bacteria which were known to be involved in this study were enterococci, lactobacilli, clostridia and *Escherichia coli*. Likewise, Tange (95) reported the production of riboflavin by cecal bacteria of the rat. Shourie and Swaminathan (89) reported that rats on low and high nicotinic acid-containing diets excreted approximately the same amount of nicotinic acid in their feces. On low nicotinic acid intakes (5 to 22 μg per day), as much as 60 μg per day were excreted while on high nicotinic acid intakes (1,250 μg per day), about 85 μg per day were excreted. The nicotinic acid content of the liver, muscles and blood were strikingly similar in rats on the two diets with highly different amounts of this factor. Hence, there appears to be a certain amount of destruction or binding of nicotinic acid if present in large concentrations.

The destruction of various vitamins by bacteria *in vitro* is now a well established fact. The classical example is that of decomposition of vitamin C by common intestinal bacteria (106, 107). Koser and Baird (49) isolated 26 cultures from soil and water which had the ability to use nicotinic acid as the sole source of carbon. Microbiological destruction of riboflavin, pantothenic acid and thiamine has been reported (27, 61, 101). Koser's (48) discussion of "Growth Factor Destruction" elaborates further on this question. A further investigation by Reid (79) has shown indirectly that ascorbic acid when injected intraperitoneally in the guinea pig is found in considerable amounts in the contents of the stomach and small intestine, but appears in significantly smaller concentrations in the cecum and large intestine. This evidence appears to indicate that destruction of vitamin C occurs in the cecum and large intestine of the guinea pig, and that such destruction may probably be attributed to the microbial population of the intestines. Furthermore, it seems plausible that intestinal destruction of certain other of the known vitamins can and does occur. This, no doubt, will be revealed by experimental work in the near future. It is to be hoped that more direct means will be employed to prove the occurrence of microbial destruction of vitamins in the gastro-intestinal tract of mammals.

It has been demonstrated, both *in vivo* and *in vitro*, that bacterial destruction of a vitamin does occur and hence this activity may play an important rôle in the vitamin balance of the animal host. Furthermore, it has been indicated that vitamin synthesis in the intestinal tract is highly susceptible to the composition of the ration (this will be elaborated upon further in the next section) and may proceed to greater or lesser degrees depending on the particular location within

the tract. Additional evidence of the delicate balance of the biological systems occurring in the intestines was compiled by Martin (57) and Woolley (102). The former noted that when the vitamin B-complex of a synthetic ration was supplied as riboflavin, thiamine, nicotinic acid, pyridoxine, choline and Ca pantothenate, mice grew well. If para-aminobenzoic acid (PABA) was included in the ration, the mice developed a deficiency syndrome not unlike that of a pantothenic acid deficiency; this deficiency could be corrected by the addition of inositol to the diet. Conversely, if inositol was incorporated in the ration, a PABA deficiency developed. Martin postulated that inositol may stimulate proliferation of pantothenate-destroying microörganisms and that PABA may inhibit them. Woolley, also working with mice, found that an inositol-free synthetic ration supported good growth of most of his animals. However, in the absence of pantothenic acid the mice developed alopecia (loss of hair), a manifestation of inositol deficiency. The inositol-synthesizing mice yielded mixed cultures from their droppings which were able to form considerable amounts of inositol—enough to bring about recovery of mice affected with alopecia. On the other hand, such mixed cultures as were isolated from the deficient mice synthesized very little inositol. An isolate of *Escherichia coli* was found to synthesize very little inositol and the presence of gramicidin (an inhibitor of gram-positive bacteria) had no effect on the degree of synthesis by mixed cultures from alopecia-free mice.

A further factor that may have to be considered in future nutritional studies is that of the rôle of vitamin analogues (84, 103, 104, 105). A recent report by Dreizen *et al.* (18) has shown that antimetabolites of nicotinic acid, pantothenic acid, and thiamine are able to depress growth and acid production of *Lactobacillus acidophilus* in a synthetic substrate. However, little is known of the natural occurrence of vitamin analogues and until this is determined, such studies may have to be deferred.

An excellent study by Mitchell and Isbell (65) has shed some light upon the degree of vitamin synthesis in the ceca of rats, the site of absorption, and the availability of certain B-complex vitamins. They studied synthesis of thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, biotin, inositol, and folic acid in rats eating lean beef with and without lactose. By removing the ceca of rats, centrifuging the contents and thus separating solids from the liquid, they were able to determine the amount of binding of the various vitamins within the bacterial cells. They found that inositol, thiamine, riboflavin, nicotinic acid and pantothenic acid were tied up in the cells to a considerable extent whereas the other members of the B-complex studied would readily diffuse from the cells into the medium. To determine the site of absorption, they took sections of the saline-washed walls of the stomach, upper small intestine, lower small intestine, cecum and colon, ground them immediately upon removal, and extracted them with boiling water. These water extracts were then analyzed for certain B vitamins (folic acid, biotin and pantothenic acid). They found that there was absorption of these B vitamins at every level of the tract but that it was considerably greater in the walls of the cecum. Since the amounts of B vitamins in the

cecal wall extracts were similar in magnitude to those values obtained for the medium surrounding the bacteria, they believed that there exists a more or less free passage of vitamins from bacteria in the cecum into the animal tissues. The action of the two diets differed in numerous respects, the main one being that the diet with lactose resulted in a slightly greater synthesis of most of the vitamins in the cecum. From their extensive study, Mitchell and Isbell calculated the approximate percentages of the dietary intake of B vitamins supplied by intestinal synthesis: thiamine, 8 to 18; riboflavin, 5 to 19; nicotinic acid, 1.8 to 3.5; pantothenic acid, 11 to 58; pyridoxine, 130 to 230; biotin, 230 to 430; inositol, 0.08 to 0.1; and folic acid, 67 to 71 per cent. Figures over 100 per cent indicate that more of the vitamin was synthesized than was ingested. Apparently, certain synthesized vitamins are more available to the animal than others according to this study. Unfortunately, Mitchell and Isbell did not attempt a study of more than two rations, but one might anticipate that the availability of the various B vitamins, *i.e.* the degree of retention within the bacterial cells, might vary considerably according to the ration employed, and the presence or absence of carbohydrate.

Gall and her co-workers (29, 30, 31) have reported on a study involving two highly inbred strains of mice differing in their degree of requirement for riboflavin and pantothenic acid; strain C_{57} is black and possesses a high amount of resistance to mammary tumors whereas strain A is albino and is very susceptible to mammary tumors. Strain C_{57} was found to require less riboflavin and pantothenic acid in its diet than the albino strain. These workers felt that this nutritional difference in the two strains of mice might be reflected in unlike bacterial floras, and that members of the flora from the two strains of mice might show a great enough difference in their respective abilities to synthesize the vitamins in question that such a difference could explain the dissimilar nutritive needs of the mice. In report II, they studied the predominant bacteria from the lower small intestine and cecum of the two strains of mice (they believed that the lower bowel is the likely area for absorption of synthetic products liberated by bacteria) on three rations: a stock ration of Purina Laboratory Chow; ration 101, an incomplete synthetic ration with dextrose as the carbohydrate; and ration 133, which was more complete than 101, and incorporated dextrin as the sole carbohydrate. They found each ration to result in a characteristic bacterial flora in the lower intestines consisting of one or more specific morphological types. The stock ration resulted in the highest bacterial counts in the cecum; the ceca of animals on this diet were larger. In their third paper, they reported isolation of bacteria from the highest three dilutions of the intestinal contents in order to obtain representatives of the predominating types and to determine their ability to synthesize riboflavin, niacin, biotin, folic acid and pantothenic acid. No cultures were identified except coliforms and their methods of assaying for synthesis of the above-mentioned vitamins were semi-quantitative in nature. Hence, no revealing conclusions could be drawn. In their final paper, a basis for explaining the nutritional differences in the two strains of mice was reported. On a purified ration, it was found that the total weight of the cecal contents of the two strains

was significantly different, although the bacterial count per gram of cecal material was essentially the same for both mouse strains. Strain C_{57} had the greatest total amount of cecal contents and strain A the least. On this basis they postulated that C_{57} actually possessed the largest total numbers of cecal bacteria and therefore greater amounts of vitamins were synthesized and absorbed in mice of this strain than in strain A. This explained their results admirably inasmuch as strain C_{57} possessed the lowest requirement for riboflavin and pantothenic acid. When the two strains of mice were maintained on a stock ration, these differences in cecal contents were not apparent. It is indicated from the results of Gall, *et al.*, that not only is there a wide variation between the nutritional requirements of various species of animals, which in certain instances can be explained on the basis of intestinal flora, but that there may also occur variations in the nutritional needs of strains of animals within one species that may be a function of intestinal biosynthesis.

It was not until the past decade that our knowledge of the various vitamins, particularly members of the water soluble B-complex, was sufficient to permit the use of highly purified, chemically defined rations, commonly referred to as "synthetic rations." According to Elvehjem (20), "Before 1940 any attempt to grow rats on a vitamin-B-complex-free ration supplemented with the crystalline vitamins then available resulted in complete failure." Within a short time it was discovered that the addition of pantothenic acid, thiamine, riboflavin, niacin, pyridoxine, and choline as the only B vitamins to purified rat rations resulted in good growth and reproduction. Elvehjem, and no doubt many other nutritionists, was not convinced that these six members of the vitamin B-complex were the only ones required by the rat, but rather were the B vitamins synthesized to the least degree by intestinal microörganisms—a view well substantiated by Mitchell and Isbell (65). Other vitamins apparently were needed by the rat but intestinal synthesis of them was so great that their presence in the ration was not a prerequisite.

Such a theory was difficult to substantiate until Marshall, *et al.* (56) reported the bacteriostatic effect of the poorly absorbed sulfonamide, sulfaguanidine, on intestinal bacteria (especially coliforms). Therefore, an approach was suggested whereby it might be determined whether or not intestinal synthesis of other factors was occurring in the rat as a result of subduing the activities of some intestinal microörganisms. The response to this suggestion was immediate, particularly by workers at the University of Wisconsin, the National Institute of Health, and the Johns Hopkins University. Within a few years' time it was discovered that incorporation of poorly absorbed sulfonamides in the ration resulted in deficiencies corrected by para-aminobenzoic acid, biotin, folic acid and vitamin K. Almost without exception, a considerable reduction in the numbers of coliform organisms accompanied the presence of certain sulfonamides in the diet. The report of Gant, *et al.*, (32) indicated that the coliform flora may gradually re-establish itself in the presence of sulfaguanidine and sulfathiazole, but Miller (62) found that neither a "sulfa-resistant" nor a "sulfa-sensitive" strain of *E. coli* was able to synthesize as much folic acid in the presence of sulfathiazole as in

its absence. Evenson, *et al.* (25) found not only the coliform flora to be affected by use of sulfathiazole in rat rations, but that lactobacilli also were reduced in numbers by the presence of the drug.

For a complete review of work reported on the use of sulfonamide drugs in nutritional and bacterial flora studies it is suggested that the reader consult Evenson (24) and the excellent, comprehensive review by Daft and Sebrell (15). Undoubtedly the use of bacteriostatic agents which are not harmful to the host is becoming a useful tool in determining the extent of vitamin synthesis by intestinal microörganisms, and the exact nutritional requirements of various animals, including humans.[3] This type of study is analogous to attempts to raise animals free from intestinal organisms, although the latter would appear to be the more ideal approach to the problem of dependence of animals on synthetic products elaborated in their intestinal tracts by microörganisms. However, progress in raising sterile animals has been slow, probably because there is synthesis of as yet unidentified growth factors which must be present in rations of bacteria-free animals for them to survive and grow.

In this section, an attempt has been made to cover the more important reports leading up to the undeniable significance of intestinal microörganisms in the nutrition of the host animal. However, the literature is extremely voluminous, and a complete coverage would be unwieldy and perhaps confusing. Therefore, it is advised that the reader supplement this summary by consulting several more extensive reviews (15, 20, 21, 22, 45, 46).

THE EFFECT OF VARIOUS CARBOHYDRATES ON THE INTESTINAL FLORA AND UPON NUTRITION

As a result of the important discovery of refection (discussed in the previous section), attention was directed to the rôle of the carbohydrate component of the ration in intestinal synthesis. The effect of lactose and lactose-containing foods upon the intestinal flora of experimental animals was known for a considerable time (81) before the observation of refection. It was known that a low carbohydrate, high protein diet resulted in a flora deficient in lactobacilli and containing large numbers of coliforms and putrefying types of bacteria. By incorporation of more carbohydrate, especially lactose, and reducing the protein in the diet, it was shown that the numbers of lactobacilli would increase and putrefying microörganisms would be considerably reduced in numbers. The necessity of starch for production of refection offered a clue as to the rôle of carbohydrates in nutrition.

Mitchell (63, 64) reported that dextrin and starch supplemented with the usual components supported the best growth of rats, while maltose and sucrose were fair, and lactose poor in this respect. She found that by using mixtures of corn starch and lactose (60 per cent total carbohydrate), a ration containing these carbohydrates in a ratio of 1:1 resulted in growth as satisfactory as when all the carbohydrate in the diet was starch. Any ratio in favor of lactose resulted in

[3] It must be realized, however, that bacteriostatic agents may do more than just inhibit growth of certain vitamin synthesizing organisms in the intestines.

reduced growth responses. She also noted that as the per cent starch was increased in the ration, the per cent total carbohydrate remaining in fecal droppings decreased. Apparently lactose was less readily assimilated in the rats she used than was starch. Whether this preferential action on starch was a reflection of microbial activity, or of an insufficiency of normal alimentary lactase, or, more probably, a delicate combination of both of these factors, cannot be determined from Mitchell's work. Such information would be highly useful.

Guerrant, *et al.* (35, 36, 37, 38) made a series of studies which elaborated on Mitchell's results. They studied rats on rations deficient in vitamins "B" and "G" and observed an increased demand for these deficient factors when dextrin was replaced by sucrose, lactose, glucose, or commercial corn starch. Thus, dextrin encouraged a vitamin-synthesizing intestinal flora. These workers concluded that the difference between the carbohydrates was due to the fact that mono- and disaccharides and starch were more quickly assimilated than was dextrin; thus, with dextrin, a residual amount of carbohydrate was available for synthesizing bacteria in the lower regions of the tract (they found the greatest synthesis to occur in the cecum). To test their hypothesis, they determined the reducing intensity, expressed as milli-equivalents (ME) of glucose, of cecal contents of rats on the deficient ration with various carbohydrates. Reducing values of from 43 to 80 ME were obtained from cecal contents of rats on starch, lactose, glucose and sucrose. However, values obtained from rats on dextrinized corn starch ranged from 292 to 327 ME. These results, unfortunately, have not been further investigated, and it would be interesting to determine the necessity of utilizable carbohydrate for the elaboration of members of the vitamin B-complex by bacteria of the intestinal tract.

Morgan, *et al.* (67) studied the relationship between the type of dietary carbohydrate and deficiencies of riboflavin, vitamin B_6 (pyridoxine) and the filtrate factor (pantothenic acid) in rats. They reported that lactose favored synthesis of riboflavin and pyridoxine, corn starch favored synthesis of pantothenic acid, and sucrose favored synthesis of none of these three members of the B-complex. Rats on the deficient diet containing starch grew best although sometimes they developed a dermatitis. In a similar study (94, 95), it was found that sucrose was very effective in producing severe riboflavin and B_6 deficiencies in rats, manifested by the appearance of cataracts and a dermatitis, respectively, whereas corn starch was beneficial, although the animals grew poorly. It was further noted that rats on B-deficient rations fed feces from lactose- and dextrin-fed rats grew normally, but that feces from rats consuming any other carbohydrate were ineffective in alleviating the deficiency syndromes. The physical appearance of fecal droppings from rats fed lactose or dextrin was normal, while those from rats on sucrose, glucose or starch diets were hard, poorly formed, and small. The ceca of autopsied rats fed lactose or dextrin rations were distended and well filled, while those from animals on one of the other carbohydrate diets were contracted and rather empty. Riboflavin was isolated only from feces of rats fed lactose- or dextrin-containing rations, and it was believed that these two carbohydrates favored the development of a synthesizing flora.

Despite the universal belief that carbohydrates are essential in the ration (22), Follis and Straight (26) published a brief report upon the occurrence of adequate growth of rats on a purified ration devoid of carbohydrate. This report seems not to have been substantiated, and until it is, any further comment is reserved. Unfortunately, they reported no bacteriological studies on these animals.

Erschoff and Deuel (23) found rations containing lactose or beta-lactose to produce alopecia and diarrhea in rats on presumably "complete" diets. Corn starch, sucrose and galactose in the same rations supported good growth with no evidence of vitamin deficiencies. Their results do not entirely agree with those of other investigators already mentioned. Mannering, *et al.* (55) found diets deficient only in riboflavin to be in every way adequate for rats if the carbohydrate component were either dextrin or starch. Isocaloric replacement of the carbohydrate with lard resulted in riboflavin deficiencies as did the use of cellulose, sucrose or lactose. Their results led them to conclude, as did Guerrant, *et al.*, that incomplete digestion of dextrin or starch allows some of the carbohydrate to reach the cecum where it can be utilized by vitamin-synthesizing bacteria. On the other hand, sucrose probably is entirely assimilated before peristalsis brings the ingesta down to the large intestine and cecum, where the greater share of synthesis presumably occurs. Results indicating beneficial effects of lactose have been announced by Schweigert, *et al.* (88) who noticed that lactose served as a better stimulant for the synthesis of thiamine and riboflavin than dextrin, but that the latter was more stimulatory than sucrose.

Bartree, *et al.* (3) obtained evidence indicating the stimulatory effect of available sugar upon the intestinal flora. They found that when three pounds of glucose were fed to a cow in addition to the customary feeding of hay, the direct bacterial counts in the rumen soon increased 100 per cent over what they had been. The desirability of a simultaneous study on vitamin synthesis is indicated by these results and might provide some explanation for the differential effect of various carbohydrates upon intestinal synthesis. There is good reason to believe that the "synthesizing areas" of the intestinal tract—the cecum and the large intestine—behave similarly to such a stimulus.

Many reports in the literature indicate the superiority of dextrin over other carbohydrates in promotion of intestinal synthesis. Luckey, *et al.* (54) found that low levels of dietary folic acid were in no way impairing to chicks, provided corn meal or dextrin was the source of carbohydrate. Sarma, *et al.* (86) found dextrin to encourage pyridoxine synthesis in rats on a B_6-deficient ration; glucose and sucrose were not stimulatory. An extensive study by Skeggs and Wright (90) showed that lactose failed to promote growth or survival of rats on a complete synthetic diet. They found dextrin-fed rats to excrete appreciably more of all the B vitamins, except riboflavin, than did rats on sucrose, cerelose, lactose or corn starch. Such rats also stored more pantothenic acid than did rats on any of the other carbohydrates. Their study also embraced work on the effect of succinylsulfathiazole on rat nutrition. They found that rats developed a combined folic acid and biotin deficiency when fed succinylsulfathiazole in 2 per cent amounts no matter which carbohydrate was employed in the ration. It was

shown that the drug produced a marked decrease in the numbers of *E. coli* but that neither succinylsulfathiazole nor the type of carbohydrate appeared demonstrably to alter the kinds or numbers of other bacteria in the intestinal tract. A recent study by Teply, *et al.* (97) further strengthened the already strong evidence concerning the stimulatory effect of dietary dextrin on intestinal synthesis. When fed a ration deficient in niacin and folic acid, rats ingesting dextrin as their sole carbohydrate synthesized a considerable amount of these two vitamins, especially in the cecum, but rats fed the same ration with lactose instead of dextrin developed homologous deficiency syndromes. They also found that addition of excess niacin increased the amount of folic acid recovered in the cecum; conversely, addition of excess dietary folic acid increased the amount of niacin recovered in the cecum.

Nath, *et al.* (69) have reported that the cecal contents of rats on a lactose-containing diet had greater total numbers of bacteria, more coliforms, and more lactics than cecal contents of rats on sucrose or dextrin-containing diets. Dextrin in the diet was found to favor higher numbers of coliforms in the cecal contents than sucrose. The aerobic and anaerobic plate counts, as well as the numbers of coliforms, were found to be decreased in the ceca of rats fed sucrose diets containing a high level of corn oil (28 per cent). However, supplementation of the high fat diet with reticulogen was found to counteract the inhibitory action of corn oil upon the growth of cecal microörganisms.

In an unpublished study by Krehl and Carvalho (Elvehjem and Krehl, 22), the use of dextrin in pantothenic acid-deficient diets greatly improved rat growth over that obtained with sucrose; this result could be correlated with higher levels of this vitamin in cecal contents, liver and muscle tissues. Krehl and Carvalho also found that biotin deficiencies were delayed in the rat when dextrin was used instead of sucrose as the only dietary carbohydrate "despite the fact that the dextrin-fed rats grew better, which would tend to increase the demand for biotin."

The latter work was well substantiated and enlarged upon by Couch, *et al.* (14) in their study on biotin deficiency in the chicken. A synthetic ration deficient in biotin and differing in its carbohydrate component was fed to laying pullets. The rations employed were as follows: (a) sucrose + basal, (b) dextrin + basal, (c) lactose + sucrose + basal, (d) sucrose + basal + biotin, (e) whey + sucrose + basal, and (f) a practical grain ration to serve as an additional control. Four hens were maintained on each of the six rations. It was found that the hens fed the dextrin diet synthesized adequate amounts of biotin in their intestinal tracts since their egg production was normal, the biotin content of the eggs produced was nearly equal to that of hens on the grain or "complete" synthetic diet (ration d), and hatchability of eggs from these hens was normal. In these respects, sucrose, whey, and lactose were poor. A concurrent study on these hens by Johansson, *et al.* (43) showed that birds on the sucrose ration had a fecal coliform flora almost too low to be detected, whereas dextrin-fed hens were found to have a very large coliform flora in their fecal droppings. Lactic acid bacteria were found to occur in the greatest numbers in the feces of hens fed

lactose- and whey-containing diets. It seems probable that the favorable effect of dextrin in biotin deficient rations for chickens lies in the establishment of conditions in the intestinal tract favorable for synthesis of biotin. Actually, there might have been no important qualitative or quantitative differences in the intestinal flora of these birds on the various diets if one accepts the well-founded theory that dextrin is less rapidly assimilated than other dietary carbohydrates. The mere presence of significant amounts of fermentable carbohydrate in the lower intestine may be a prerequisite for vigorous biotin synthesis; if the amount of carbohydrate remaining after assimilation is too little, biotin synthesis might be hindered.

Actually, there have been few concerted attempts to design experiments from which data could be derived pointing to the exact nature of the influence of the type of dietary carbohydrate on the elaboration of nutrilites within the gastrointestinal tract. A wealth of indirect information has accumulated since the advent of synthetic rations and there is, no doubt, a significant relationship between the degree of intestinal biosynthesis and the accompanying carbohydrate in the diet. It appears, therefore, that the nature of dietary carbohydrate is very important when the degree of intestinal vitamin synthesis by microorganisms is considered. The greatest share of evidence points to the more complex carbohydrates as being the most stimulatory in vitamin synthesis, and the simplest carbohydrates as being the least stimulatory.

More informative studies similar to those of Mitchell and Isbell (65) and of Benesch (4), which have been reviewed in the first section of this paper, would be welcome and should contribute materially toward our understanding of the basic rôle of intestinal microörganisms. We know that there are numerous influences upon the types, numbers and activities of intestinal microörganisms, e.g., diet (which in itself is an extremely variable factor), pH, Eh, the animal species involved, the normal digestive activity, and possibly certain other factors (surface tension, mineral concentration, natural metabolic antagonists, synergisms, and age of the animal). Therefore, the ecological system occurring in the alimentary canal must be complex and delicately balanced, and lends itself poorly to accurate scientific study. Efforts to raise animals free from intestinal bacteria, though sometimes successful, do not necessarily prove that an intestinal flora is superfluous for proper nutrition. It must be realized that such animals are maintained on the most nutritionally adequate diet that it is possible to provide. In all probability, most animals, and human beings in particular, are rarely provided with a completely balanced diet, and apparently rely upon synthetic activities of their intestinal microörganisms to provide many of the deficient growth factors.

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THE RELATIONSHIP BETWEEN BACTERIA AND MYXOPHYCEAE

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CONTENTS

An attempt to clarify the relationships between Bacteria and Myxophyceae[2] hardly needs an excuse. If one or other reader were induced to contribute facts or considerations that constituted a step forwards, it would be the best justification for this review. The imperfect state of the conclusions reached is partly due to the unequal value of the literature which is so extensive and scattered that the writer found it impossible to use it as fully as he had intended.

The errors often encountered consist in the reference of certain microörganisms to Bacteria or Myxophyceae or their interpretation as intermediate forms between these two large classes on the basis of superficial resemblances. In such minute and often little differentiated organisms detailed study and steadily improved methods of investigation are requisite in order to determine what characteristics are of taxonomic importance. Certain errors have persisted long after

[1] I wish to express my sincere thanks to Dr. B. N. Singh (Rothamsted) and Mr. E. A. George (Cambridge) for reading the manuscript and to Prof. F. E. Fritsch for criticism and corrections.

[2] "The name Myxophyceae has priority over the possibly more suitable designation Cyanophyceae. The class is also often named Schizophyceae or Phycochromaceae." (Fritsch, 1945, p. 769). The popular name is Blue-green Algae.

the means for their removal were available owing to the lack of trained investigators sufficiently interested in theoretical questions of general microbiology. It is the aim of this article to show what conclusions can be reached with the help of the available facts, and to indicate where renewed investigations would probably yield far-reaching results.

The idea that Bacteria and Myxophyceae were closely related originated from Cohn (1853). He had discovered apochlorotic Algae, i.e., unpigmented organisms obviously derived from pigmented ancestors by the loss of chromatophores. This led him to compare other colorless microörganisms with pigmented ones, and to group together in his system of classification those organisms of either kind that showed the greatest similarity. At that time it was an established principle to use only morphological features for taxonomic purposes, while color was regarded as a secondary, merely physiological property. In Cohn's day it was easier to find colorless counterparts to pigmented forms since both were far less known.

Cohn's further aim was to find a means of arranging the chaotic host of Bacteria by differentiating them by their shape and behavior, and he thus became the father of scientific bacteriology. He expressed his opinion of their status in the following words: "The Bacteria (Vibriones) appear all to belong to the Vegetable Kingdom, showing a near and immediate similarity to obvious Algae." The Algae he had in mind were the Myxophyceae which were referred to the Algae because of their possession of assimilatory pigments. Eighteen years later Cohn (1871/72) published his natural system of the cryptogams. He recognized that the Bacteria had no relation to yeasts and moulds, as was then often held, and referred them to a new group, the Schizosporeae, to which he subsequently gave the more suitable name Schizophyta. The latter comprise the Schizophyceae (Myxophyceae) and the Schizomycetes (Bacteria). In a communication on the "Verwandtschaftsbeziehungen der Bacterien" (1875) Cohn gave a more detailed account of his views. The Bacteria were considered to be near, though primitive allies of the Myxophyceae, adapted to a saprotrophic or parasitic mode of life. While refuting any relation to Animals or Fungi, Cohn stated that "The Bacteria are at the lower end of the line of Myxophycean evolution," although a closer relation between certain forms of the two classes was assumed.

An inclination to include unpigmented forms among the Algae wherever possible became widespread. Rabenhorst (1864/68) referred the entire Bacteria to the Myxophyceae; thus *Sarcina* is grouped with the Chroococcaceae, *Bacillus*, *Spirillum*, *Sphaerotilus*, etc., with the Oscillariaceae. For some time Cohn's views were widely accepted, in part owing to the influence of Sachs' textbook (1868 and later) which was predominant (cf. Pringsheim 1932b, pp. 184 seq). A few biologists, however, opposed early. Van Tieghem (1880, p. 177) in his communication on the "Affinités des Bactériacées et des Phycochromacées" claimed to have found green Bacteria which were not Myxophyceae, and colorless Myxophyceae which were not Bacteria. He therefore emphasized the wide gap between the two classes and mentioned as the main differentiating features the mode of spore-formation in the Bacteria and the pigments associated with chlorophyll in the Myxophyceae, although their nature was not known at that time.

De Bary (1884) also opposed Cohn's system of classification. Under his influence the "Fungi" were again regarded as a distinct group, separate from pigmented plant organisms. Some authors even went so far as to regard as a "Fungus" every colorless non-flowering plant. At the present day pigment-free forms recognizable as belonging to a definite group of chlorophyll-containing plants are referred to that group, while of others it can only be said that they appear to be descended from a long series of colorless ancestors (Pringsheim, 1941).

This point of view was initiated by Cohn himself. At first (1875, p. 202) he combined colorless and pigmented groups quite logically because he believed that "genera founded on pigmentation have only conventional value." Such differences he regarded merely as physiological and therefore not significant for systematic purposes. By degrees, however, he became aware of the difficulties of grouping together Schizophyceae and Schizomycetes. The flagella detected in motile bacteria in particular made him cautious. "The existence of flagella in *Spirillum volutans*, which constitute an approach to the Flagellata renders the natural position of these organisms doubtful because no Oscillatoriacea (i.e., motile Myxophyceae) possesses flagella" (Cohn, 1871/72, p. 187). Thus he very early recognized one of the main obstacles to a close affinity between Bacteria and Myxophyceae, and it is surprising that van Tieghem does not refer to it. Cohn was followed by his collaborator Schroeter (1889, p. 141), who again emphasized the endogenous spore-formation of Bacteria as a feature differentiating them from the Myxophyceae.

Gradually more and more opinions against the habitual grouping together of Bacteria and Myxophyceae found expression, so that eventually it was almost completely abandoned but for some nomenclatural relics.

Pascher (1931, p. 330) is outspoken in his rejection of a common taxonomic unit embracing the Bacteria and the Myxophyceae. Although he groups the Cyanophyta (Myxophyceae) together with the Schizomykophyta (Bacteria) as "Plantae holoplastideae" by contrast to other vegetable organisms or "Plantae euplastideae," he regards them as completely independent tribes. "The fact that there are also colorless blue-green Algae by no means justifies the assumption that the Bacteria are an apochromatic line parallel to the Myxophyceae. Myxophyceae and Bacteria (the latter in the real [eigentlichen] meaning of the word) at present possess no relation to one another. I share completely Geitler's view. Very diverse structures are still accepted as Schizomycetes: true Bacteria, apochromatic blue-green Algae, probably also very reduced Fungi, and possibly organisms of yet another origin." Which "Bacteria" Pascher would include in the last two instances he does not say.

Bháradvája (1940, p. 203) devotes a chapter to the "Relation between the Myxophyceae and the Thread-bacteria," stating that "no convincing evidence has yet been available to lend support to any affinity between them."

Stanier and van Niel (1941), on the contrary, come to the conclusion that the Myxophyta (Myxophyceae) resemble the true Bacteria far more closely than they do any of the other algal groups (p. 450). They believe that Bacteria and blue-green Algae have originated from common ancestors and summarize their

common characteristics as follows: (1) absence of true nuclei, (2) absence of sexual reproduction, (3) absence of plastids (p. 449).

These three features are employed to define the "Kingdom of Monera" (p. 456), a term Stanier and van Niel prefer in place of Cohn's Schizophyta. The entirely negative characteristics upon which this group is based should be noted, and the possibility of a convergent evolution of the two classes be seriously considered. Stanier and van Niel's conclusion (p. 450) that "it seems at least possible that the blue-green Algae of the *Chroococcus* type have developed from the Eubacteriales as a second photosynthetic line, at first paralleling morphologically the Purple Bacteria, but undergoing in the course of time a far more complex morphological evolution", is so interesting that it will be considered more fully later.

Fritsch (1945, p. 860) again is not in favor of grouping Bacteria and Myxophyceae together. "With the true Bacteria, despite an analogous lack of cell differentiation, any close affinity appears to be ruled out." In relation to *Beggiatoa* and *Thiothrix* he says: "The facts . . . suggest the possibility that these types belong to a plurality of evolutionary lines diverging from a remote ancestry, from which the Myxophyceae also arose." These cautious statements are characteristic of the modern reluctance to admit phylogenetic relations without very distinct evidence.

Dubos, who may be cited as a representative of the bacteriologists, is less cautious when he says (1945, p. 6): "One finds among bacteria organisms which show strong resemblances to certain of the blue-green algae, to the fungi, to the myxomycetes, or to the protozoa, and which can only be distinguished from these microorganisms by their much smaller size." It may be surmised that he has in mind the Beggiatoaceae, the Actinomycetes, the Myxobacteria, and the Spirochetes, but the statement as to the nature of the differences must be rejected. The Beggiatoaceae are not smaller than their pigmented counterparts, the Oscillatoriaceae; on the contrary *Beggiatoa mirabilis* is the largest member of both families, and *Achromatium* may likewise be very big (cf. p. 68). The other groups, which Dubos may have had in mind, exhibit no more than a superficial resemblance to Bacteria and to the organisms he mentions.

Dubos continues: "Some investigators have looked upon bacteria as a primitive, homogeneous group from which higher types have arisen. It appears more likely, however, that these microorganisms constitute a heterogeneous group of unrelated forms. Even among the Eubacteriales—the so-called true bacteria—one finds strange bedfellows, such as small Gram negative autotrophic organisms, the Gram positive proteolytic spore-formers, the acid-fast bacilli, which differ so profoundly from each other in metabolism, structure, and even mode of division as to have little in common except microscopic dimensions." This statement should have been more clearly substantiated. If there are any investigators of weight, who regard the Bacteria as a homogeneous group, they should have been named. The differences in metabolism are now known not to be so great as to justify wide taxonomic separation. What differences in cell-division has Dubos in mind? If he is certain of the gaps between the various

groups of Eubacteria, why does he not submit a scheme of classification for which bacteriologists are waiting?

I. TAXONOMY OF BACTERIA AND NATURAL RELATIONSHIPS

The answer to the question whether there is an affinity between Bacteria and Myxophyceae depends so much on the delimitation of these groups that some space must be devoted to a discussion of these matters. In the case of the Myxophyceae it is relatively easy to define the class. All authorities agree that they are a natural group without affinities to other algal classes (Fritsch, 1945, p. 859), except a most doubtful one to the Bangiales, and differing from the other microörganisms under discussion in possessing true chlorophyll. The so-called Chlorobacteriaceae or Cyanochloridinae (Geitler and Pascher, 1925) alone remain of doubtful affinity (cf. p. 80).

The taxonomy of the Bacteria in the widest sense is much more difficult. It has unfortunately been customary, in order to secure any kind of practical arrangement, to divorce the classification of the forms from the problem presented by their natural grouping based on phylogenetic considerations. Hence the deplorable state of bacterial classification (Pringsheim, 1923), severely criticized again by Stanier and van Niel (1941, p. 439).

The paucity of differentiating morphological features makes it imperative to employ every available character in order to describe the vast number of bacterial species (Pringsheim, 1923, p. 364). The main problem is to define the importance of these characters in relation to the distinction of larger and smaller taxonomic entities respectively. To the morphological characters provided by cellshape, mode of flagellation and presence or absence of spores, which are used in the first instance, the gram stain has been added as a characteristic of equal importance (Pringsheim, 1923, p. 375). This latter has meanwhile been found to "divide the bacterial world into two groups which differ profoundly" (Dubos, 1945, p. 294).

By making use of all these means the genera *Pseudomonas*, *Vibrio*, and *Spirillum* have been recognized as allied. These genera were removed from the Bacillaceae and called Spirillaceae (Pringsheim, 1923, p. 376). The latter might well, however, be regarded as of higher rank than a family (cf. p. 81). Why later authors took *Pseudomonas* instead of *Spirillum* as the type-genus and called the family Pseudomonadaceae (Kluyver and van Niel, 1936, p. 388; Stanier and van Niel, 1941, pp. 441, 453) is not apparent. Bergey (1939, p. 82) attributes the name Pseudomonadaceae, used in that sense, to Winslow *et al.* (1917, p. 555); but these authors use it in a narrower sense, with the exclusion of *Vibrio* and *Spirillum*. Moreover, the group Rhodobacteriales was established to include the photosynthetic bacteria as opposed to the colorless sulphur bacteria (Pringsheim, 1923, p. 374), with which they had long been associated (Stanier and van Niel, 1941, p. 448).

Most recent authors, though accepting these propositions, do not go far enough in utilizing all available evidence of differentiation or of affinity among bacteria for taxonomic purposes. When the taxonomy of other groups of organisms was

in an embryonic state comparable to that of bacteria at the present day the first advance always consisted in singling out assemblages of species which were obviously closely allied. Between such groupings sets of more or less intermediate forms could be recognized, until there remained only a few aberrant forms. A similar procedure has been adopted in the Bacteria, but often without sufficient consciousness of the necessity of bringing together the species with the largest number of common features. Otherwise such unnatural groups as the Thiobacteriales, the Trichobacteriales, and the Caulobacteriales, would not have been established.

The Thiobacteriales are supposed to be characterized by the presence of sulphur or of red pigment or of both in their cells, without the latter forms constituting a phylogenetic bridge between the two other groups, so that this classification is neither natural nor practical. The only common characteristic of the Trichobacteriales is the filamentous habit, although they do not comprise all filamentous bacterial forms. The only common features of the Caulobacteriales (Henrici and Johnson, 1935) are their stalk-like exudations, the nature of which is unknown in most of them, and where it is known, is not the same in all cases.

Single characteristics never suffice for the creation of natural groups (Pringsheim, 1923, p. 364) and in most bacteria morphological features are inadequate for the purpose. If therefore one is not to be deceived by superficial resemblances, affinities must be tested in every possible way, physiological features being considered side by side with morphological ones, which should certainly include sporulation and flagellation. Natural and readily recognizable groups in this sense are, for instance: the Escherichia-Salmonella group; the Pseudomonas-Vibrio-Spirillum group; the Subtilis-Mycoides group; the Corynebacterium group; the Sphaerotilus group, and many more. Features regarded as characteristic of a whole group may occasionally have to be abandoned as a result of new observations, as happened when spore-forming species of *Sarcina* and *Vibrio* were found. These genera are, however, too well characterized by other features to be affected by an alteration in the diagnosis.

The difficulties of establishing a system of classification that shall serve both the practical need of a clear grouping and afford an expression of natural relationship, are due to: (1) the small size of most bacterial cells as a result of which they do not show many differentiating features; (2) the multitude of similar forms; (3) the lack of sexual reproduction and of the morphological characteristics associated with it which are of prime taxonomic importance in many other groups; (4) the frequent neglect of numberless species in favor of those of practical importance. These difficulties increase with the discovery of more and more species, because bacteriologists who specialize in certain groups do not have a sufficiently wide outlook, while those interested in the class as a whole often lack a detailed knowledge of its various groups.

The necessity for the inclusion of numerous forms, many of them more interesting for their enzymatic activity than for their natural relationship, has led to the establishment of systems of classification primarily on physiological characteristics which, although perhaps not unsuitable for the differentiation of species,

have to be used with caution. Otherwise one obtains wholly unnatural groups, embodying forms of widely differing relationship; quite a number of them could be cited.

For example the most modern classification of bacteria, that of Stanier and van Niel (1941), which is by far the best, still retains a relationship of the Myxobacteria and the Spirochaetae to the Eubacteria. The former two groups might, however, more naturally be regarded as having an origin quite different from the latter, being possibly derived from common ancestors with the Myxophyceae, and resembling true Bacteria only in shape and dimensions, a question which will be considered subsequently.

II. RESEMBLANCES AND DIFFERENCES BETWEEN BACTERIA AND MYXOPHYCEAE

1. Morphology. The Myxophyceae differ from Bacteria in a greater structural complexity and diversity. The Stigonematales possess a thallus differentiated into prostrate and erect systems of trichomes, an elaborate organization unknown in any member of Bacteria even in the widest sense accepted. In fact there are few morphological parallels between the two groups. The filaments of Bacteria are in general not comparable to those of the Nostocales; *Streptococcus*, it is true, has some similarity to *Pseudanabaena catenata*, and *Sphaerotilus* to *Lyngbya*, but the resemblances are superficial (cf. p. 77). *Beggiatoa* will be discussed below.

While bacteria, as a rule, do not form distinct colonies in nature, colonial Chroococcaceae are imitated to some degree by a few of them. A parallelism is recognizable between the blue-green *Merismopedia*, the cells of which divide along two planes and show a regular arrangement in a single tabular layer, and the purple sulphur bacterium *Thiopedia*, and *Lampropedia* among Eubacteria, in which the same arrangement is found. *Eucapsa* among Myxophyceae, which is characterized by division along three planes, has its parallel in *Sarcina* among Bacteria. *Chroococcus* divides in the same way, although the cells cohere only as groups of four or eight within mucilaginous envelopes, so that it has a certain similarity to *Gonococcus*, *Meningococcus*, and *Micrococcus tetragenus*.

Similarity between Bacteria and Myxophyceae as regards their external morphology is therefore very restricted and not really helpful in assessing their relations to one another. The fact that only the least specialized Myxophyceae, if lacking color and motility, could possibly, as suggested (Benecke, 1912, p. 244; Stanier and van Niel, 1941, p. 449), be confused with Bacteria, is not in favor of more than a very remote relationship.

Among Myxophyceae even relatively simple cell rows, like those of *Oscillatoria*, are more highly organized than bacterial filaments. The former possess differentiated terminal cells, often with a calyptra; zones of intercalary division; and defined propagatory structures in the form of hormogonia. The greater differentiation of Myxophyceae as compared with Bacteria is also expressed in the existence of protoplasmic connections between adjacent cells (Stigonemataceae), in the formation of spores in definite positions (*Cylindrospermum*, *Gloeotrichia*),

in hormogonium formation being often initiated by the death of certain cells (*Scytonema*), and in the false branching occasionally being related to the development of heterocysts (*Tolypothrix*) and in such instances being more regular than in *Cladothrix* among Bacteria.

Certain bacteria, for instance *Sphaerotilus*, *Lineola*, and *Caryophanon* exhibit the first steps towards a differentiation between cells. In the two last, instead of the periodic hormogone formation of *Oscillatoria*, there is a progressive formation of steadily deepening constrictions until that first formed and situated approximately at equal distances from either end, divides the whole trichome into two halves, within which further constrictions have arisen repeatedly between those first formed. No species of *Oscillatoria* seems to develop such constrictions which at an early stage define the place of fragmentation.

As repeatedly pointed out in the literature, spore formation also differs in the two classes. No Myxophycea produces endospores comparable to those of Bacteria. Where so-called endospores are formed, as in *Dermocapsa* and many Pleurocapsales they are different in organization from those of Bacteria, while the spores (akinetes) of Nostocales are entire transformed cells, for which there is no parallel in any bacterium.

2. Pigmentation. The cells of most Bacteria do not contain assimilatory pigments, while those of Myxophyceae possess chlorophyll a, carotins, phycocyanin, and often phycoerythrin, although a few genera are colorless (cf. p. 61). Among Bacteria the Purple and Green Bacteria contain, in addition to other pigments, green ones similar to chlorophyll (cf. p. 79), but differing in the two groups. The pigmentation does not afford any indication of relationship between Bacteria and Myxophyceae.

3. Cytology. The myxophycean cell is differentiated into two regions: an inner, the central body or centroplasm, and a peripheral one, the chromoplasm, containing chlorophyll and accessory pigments. The presence of two regions is not established beyond doubt in every case (Chodat, 1894; Geitler 1936, p. 6; and own observations). The central part of the cell is characterized by an accumulation of reserve substances and perhaps a certain degree of independence during division (Fritsch, 1945, p. 780), while the chromoplasm appears to be different mainly by its pigmentation and is not sharply demarcated. The centroplasm also often contains rod-shaped structures, simulating the chromosomes of other organisms without exhibiting the properties of true nucleins (cf. Guilliermond, 1926b). Granules giving the Feulgen reaction have also been found (Poljansky and Petruschewsky, 1929; Petter, 1933; Spearing, 1937; Delaporte, 1939, 1940) but their fate during cell division is not clear, and they seem less defined than the nucleoids of bacteria (Robinow 1944, 1945; Klieneberger 1945, 1947).

The differentiation into central and peripheral cytoplasm is wanting in the pigment-free genera *Beggiatoa* (Delaporte, 1939, who gives the older literature) and *Thiothrix* (Fourment, 1926) which otherwise have much in common with certain Myxophyceae. This lack of correspondence must not be overestimated. Fischer's conclusions (1897a, pp. 119 *et seq.*) seem still to be valid. According to

him, "the centroplasm is no more than the main body of the protoplast surrounded by the chromoplasm in which are also stored the products of assimilation."

Changes in cell-structure, somewhat comparable to the differences in cell structure between *Oscillatoria* and *Beggiatoa*, are connected with apochlorosis in various algae. The loss of pigments in *Euglena* and other flagellates, for instance, is associated with the destruction of the chromatophores, and the chondriome system also undergoes changes (Pringsheim, 1949). It is therefore not surprising that in apochlorotic Myxophyceae also the loss of pigments is connected with a reduction of the differences between the regions of the protoplast. A similar reasoning can be applied to the storage of reserve substances, which are deposited inside the chromatophore in *Chlamydomonas* but in the cytoplasm in the related chlorophyll-free *Polytoma*. Differences in this respect are likely to occur even between closely related phytotrophic and heterotrophic organisms.

The process of cell division by diaphragm-like ingrowths from the periphery towards the center is similar in both classes. During such division the daughter cells often remain joined by connections, while secondary divisions are in preparation. The parallelism in cell-division may indicate a remote affinity but may equally well be merely superficial.

In the large purple sulphur bacterium *Chromatium* the cell structure (Dangeard, 1909; Guilliermond, 1932; Delaporte, 1940, p. 42; cf., however, Fischer, 1897) resembles that of certain blue-green Algae inasmuch as the pigments are restricted to the outer cytoplasm, while the inner region resembles the centroplasm of Myxophyceae. Although the peculiar, almost homogeneous finely granulate appearance of the cell constituents increases the similarity, Guilliermond's view that *Chromatium* and its allies should be grouped among the Myxophyceae, cannot be accepted (Fritsch, 1945, p. 860), since the presence of flagella in the former is too strong an argument against such a relation. The peripheral location of the pigments in both cases may be due to the same physiological causes without a derivation from a common ancestry.

There are other differences in cell-structure between Bacteria and Myxophyceae. The former have a true cell-wall (Fischer, 1895, 1897b; Benecke, 1912, p. 90; Robinow, 1945), while the cells of Myxophyceae seem to be bounded merely by a differentiated layer of the outer cytoplasm, apart from mucilage (Geitler, 1936, pp. 25 *et seq.*; Fritsch, 1945, pp. 778, 788). Bacteria undergo true plasmolysis in hypertonic solutions. In the Myxophyceae the whole cell shrinks without the protoplasm being detached from the wall (Geitler, 1936, p. 4). Vacuoles filled with cell-sap, such as are found in higher plants and in many Algae and Fungi, are lacking in healthy cells both of Myxophyceae and of Bacteria. The former are suspected of having a gel-like protoplast although this requires substantiation (Fritsch, 1945, p. 771). It remains doubtful whether there is a resemblance in this respect to the Bacteria.

4. Physiological characteristics. Myxophyceae exhibit such varied habits and occur in habitats of such different kinds that no general conclusions regarding their ecological adaptations are possible. Species of blue-green Algae are found

wherever living organisms can exist, occurring in marine and freshwater localities, in water rich in organic substances or containing extremely low concentrations of even indispensable inorganic salts. Many are terrestrial and some of them are among the first organisms to colonize barren rock and sand; some can stand high concentrations of brine, acids, hydrogen sulphide, or carbon dioxide. In short, Myxophyceae can adjust themselves to all kinds of extremes, even to the lowest and highest temperatures any organism can endure. Such adaptations are, however, found only in certain species or groups of species. Little is known of the capacity of the majority of Myxophyceae to accommodate themselves to their environment, nor what role competition may play in the predominance of certain forms in special habitats.

In this connection motility, dealt with in the next section, and resting stages are no doubt important. Among the latter the spores (akinetes) of Nostocaceae (*Nostoc, Anabaena, Cylindrospermum*) are noteworthy. They withstand drying and can germinate after long periods of inactivity. They can also survive heating almost to boiling point and cooling to −60 C. In all these respects their power of resistance contrasts with that of the vegetative cells of the same species which are even more susceptible than those of non-sporulating Myxophyceae (Glade, 1914).

Many Myxophyceae remain alive without multiplication for long periods of nutritional deficiency despite the absence of morphologically discernible adaptations. This was observed in cultures of *Synechococcus parvus* n.sp., *Merismopedia glauca, Oscillatoria formosa, Pseudanabaena catenata*, and *Phormidium faveolarum*. When the medium becomes nitrogen deficient the cells bleach out by reduction of the chlorophyll content (Magnus and Schindler, 1912; Pringsheim, 1913; Boresch, 1913) but on addition of nitrate recover their original hue even after many months. Such faintly colored trichomes are often found in nature, and there is every reason to assume that the phenomenon is the same. Although such growths may have long ceased to multiply they can still be used to start cultures, indicating that Myxophyceae are able to survive unfavorable conditions in this way. There is also evidence that numerous species can withstand desiccation in the vegetative state.

A comparison with Bacteria with respect to these properties is difficult. Except in really barren habitats Bacteria are also found everywhere, but little is known about those species which do not occur in dense populations and fail to multiply in routine bacteriological media. Certain Bacteria are adapted to all the extremes mentioned above for Myxophyceae. Some (e.g., Sarcina) are able to resist drying, others survive in an active state without much change or visible adaptation if the conditions of multiplication, e.g., in water devoid of nutrient substances, are lacking. Bacteria can also produce spores, though these are of a different morphological nature (cf. p. 54); they are still more resistant than those of Nostocaceae. Spores are produced both by aerobic and anaerobic Bacteria. Some of the nitrogen-fixing species sporulate, without any obvious relation to ecological conditions.

In popular descriptions and in textbooks emphasis is generally laid on the im-

pressive chemical activity of Bacteria in relation to disease or the decompositon and fermentation of organic substances. Nothing of the kind occurs among Myxophyceae. In this respect the former approach more nearly to the Fungi, while Myxophyceae resemble the Algae, in correspondence with the possession of assimilatory pigments. The anaerobic fermentation, which is supposed to lead to the production of gas vacuoles in certain Myxophyceae (Canabaeus, 1928, p. 25, following a suggestion of Kolkwitz) has still to be proved experimentally. Even if colorless Myxophyceae (cf. p. 61) are included so that photosynthesis does not come into consideration, there is still a great divergence between the two classes in fermentative activity.

Actually only a relatively small number of Bacteria are conspicuous by their parasitism or the performance of large-scale chemical transformations, but it is just this minority which has provided the material for most bacteriological investigations. The great achievements thus attained must not distract attention from the fact that a large proportion, possibly the great majority of Bacteria, are very economical in their nutritional requirements and thus escape the attention of the bacteriologist.

Among Myxophyceae there are, on the other hand, a considerable number that live in an environment providing a rich supply of organic substances. Such forms are found in the mud of polluted waters, both marine and freshwater, on dead animals, feces, seaweeds, rotting wood, within the tissues of flowering plants (*Gunnera, Cycas*), or associated with other cryptogams (*Azolla, Blasia*, Lichens). Little is known of the biochemical, especially enzymatic, properties of these specialized forms, but there is some evidence that pigmented Myxophyceae, inhabiting spaces within the tissues of other plants, are able to multiply in the dark, if provided with sugar and organic nitrogen compounds (Pringsheim, 1917 Harder, 1917); certain free living species have been treated in a similar way without positive results (Pringsheim, 1913, and unpublished experiments). More species of Myxophyceae will probably be found to benefit from organic compounds in the substrate, although the mere presence of such substances should never be regarded as indicating that the organisms present are adapted to a heterotrophic or mixotrophic mode of nutrition, as has repeatedly been done (Pringsheim, 1913).

Fixation of elementary nitrogen has been established in various, otherwise unrelated microörganisms. Among Bacteria it takes place in *Azotobacter, Clostridium*, and *Rhizobium*, and in other endobiotic species. The fact that nitrogen fixation shows no relation to systematic position suggests that it may still be found in other organisms, perhaps in a less efficient form. This is also true of nitrogen fixation among Myxophyceae (Beijerinck, 1901) which so far has been established only in species of Nostocaceae (for references, see Fogg, 1942; Fritsch, 1945, pp. 871/72). This need not be a mere coincidence, but, owing to the difficulty of obtaining bacteria-free cultures, few Myxophyceae have so far been isolated in pure culture, and most of these have been lost. The pervading mucilage is generally inhabited by bacteria and much patience is necessary to eliminate these epiphytes (Pringsheim, 1946a, p. 94).

One special method of nutrition has long been regarded as the peculiar property of Bacteria, viz., chemo-autotrophy, or the utilization of chemical energy derived from the oxidation of inorganic compounds. Bacteria, which oxidize ammonia, nitrite, hydrogen, sulphur, hydrogen sulphide and other compounds of sulphur, are known to reduce carbon dioxide and to convert the organic substances thus synthesized into body-building material. *Beggiatoa*, the first organism recognized as living chemo-autotrophically (Winogradsky, 1887), is not, however, a 'sulphur bacterium', but a colorless member of Myxophyceae (cf. p. 68). This and the related genus *Thiothrix* have been shown to be autotrophic also in pure culture (Keil, 1912). The former, like many autotrophic bacteria, is also able to live on organic substances (Cataldi, 1940; and my own observations).

To sum up, there is no unbridgeable gap between Bacteria and Myxophyceae in physiological respects, but these classes have no feature in common which is not also found in other organisms. Of the two, certain Bacteria alone appear to have acquired an intensive biochemical activity and thus to consume an amount of material not required by organisms that live more economically.

5. Movement. Lower organisms exhibit two kinds of locomotion, swimming, and creeping or gliding. The mechanisms of both are still hypothetical. Swimming permits free movements through the water, while gliding requires a solid body or at least the surface film of the water as a substratum. The two modes of locomotion are mutually exclusive so that very few organisms (i.e., certain Flagellata, cf. p. 88) exhibit both, either simultaneously or at different stages of their life cycle.

So far as they possess active movement at all, Bacteria swim and Myxophyceae glide. The locomotion of Bacteria is brought about by flagella, as has been known since these organs were discovered in *Spirillum volutans*. The reasons for regarding the movements of Bacteria as caused by flagella were, for instance, discussed by Migula (1900, I, p. 110), and have never been challenged since.

Pijper (1940, 1946, 1947), however, has attempted to substitute for the concept of flagellar locomotion in Bacteria an undulating movement of the flexible body, the 'so-called flagella' being regarded as trailing strands of mucilage. In order to reduce the danger of confusion Pijper's views must be discussed here. Views analogous to his are to be found in the older literature. Nägeli (1849) believed that the flagella of algal swarmers were without significance for their movement. Van Tieghem (1879) tried to explain the movements of bacteria as being due to protoplasmic contractions, the flagella being superfluous appendages, an opinion which Hüppe and de Bary were inclined to accept.

In larger cells, like flagellates and the zoospores of algae, the flagella have long been recognized as organs of locomotion. Those of bacteria, already detected with the help of photographs by Koch (1877), were established as regularly present in motile forms by Löffler's (1889) method which was more extensively applied by Fischer (1895). These discoveries put an end to doubts as to the means of movement in bacteria.

In view of much evidence Pijper's contradictory theory is not convincing. There is no reason why the mechanism of swimming in bacteria should differ

fundamentally from that of other flagellate cells. Their flagella are not strands of mucilage, but well-defined cell organs with specific antigenic properties. They can be seen as distinct threads not only in preparations stained in various ways and in electron micrographs, but also in the living organism. In large bacteria, such as *Spirillum volutans* and *Chromatium okenii* their active beating can be demonstrated. With the help of darkground illumination bell-shaped rotation figures can be observed and their reversal watched when the cells change the direction of their movement. Various species possess a definite number of flagella, with a characteristic arrangement and of a definite length. After division the newly formed ends of the daughter rods bear shorter flagella. Flagella are present only in motile species but are found in all of them and are confined to the motile stages. If they merely represented mucilage strands it should be possible to find motile bacteria devoid of such structures.

Pijper also fails to prove that bacteria possess a mechanism which could replace flagellar action. His own photographs (1946, pl. XLVII, fig. 20) show that, contrary to his statements, bacterial cells do not change their shape during movement. The slight curvatures seen in the photographs remain remarkably constant and reappear in the same shape after every rotation. The undulation of the body which is supposed to drive the bacterial cell through the water, does not exist. Many problems connected with flagellar movement remain unsolved, but there is little promise in Pijper's approach.

The evolution of flagella is entirely obscure. Since 'amoebae' are no longer regarded as mere lumps of protoplasm nor as the most primitive organisms, there are no grounds for interpreting flagella as modified pseudopodia. There are, moreover, organisms in which ameboid and flagellated organization exist side by side without apparent morphological relation between pseudopodia and flagella. Like those of flagellates, the flagella of bacteria are cytoplasmic organs connected through holes of the cell-wall with the surface of the protoplast (van Iterson, 1947, fig. 11; Kingma-Boltjes, 1948, p. 279).

No true bacterial organism (i.e., excluding colorless Myxophyceae and Myxobacteria) exhibits gliding movement. Winogradsky (1888) and Cholodny (1926, pp. 7, 10, 14) claim that the filaments of *Leptothrix* creep out of the old sheaths and form new ones, but this gliding movement is only a matter of conjecture. Special investigations directed to that end failed to provide confirmation of such movements either in liquid media or on agar (Pringsheim 1949a, p. 461). On the other hand such bacteria, as *Proteus vulgaris* and *Bacillus sphaericus*, which move over the surface of agar plates, appear to use flagella as the means of locomotion.

Gliding movement is characteristic of Myxophyceae, and no flagellate stages have ever been observed. The movements of *Oscillatoria* and *Beggiatoa* are generally familiar, although not completely elucidated (Fritsch, 1945, pp. 800–804), but the extent and frequency of locomotion among Myxophyceae are greater than often realized.

Stanier and van Niel (1941, p. 449), after enumerating the characteristics common to true Bacteria and Blue-green Algae, say: "One major difference, how-

ever, is the complete absence of flagellar motility in the Myxophyta, whose representatives are either immotile or exhibit creeping motility. It is among the Chroococcales, the most primitive assemblage of the Myxophyta, that we find forms closely resembling the Eubacteriales. A *Chroococcus* sp., for example, would be indistinguishable from a *Micrococcus* sp. if it were to lose its photosynthetic pigments." The general argument in this paragraph is sound. It was already hinted at by Benecke (1912, pp. 31/32) and is repeated, for example, by Dubos (1945, p. 11), although he does not mention its origin. The conclusion, though, that such primitive Myxophyceae have perhaps developed from Eubacteria (cf. p. 49) could with greater probability be reversed.

It does not seem, however, that the Chroococcaceae are generally immotile. Stanier and van Niel (1941, p. 452) mention Jahn's (1924, p. 102) claim to have found creeping motility in (unidentified) unicellular Blue-green Algae, although they state that this requires substantiation. A number of other instances of movement in Chroococcaceae have become known. Fritsch (1945, p. 800) believes it to be not uncommon. Geitler (1925, p. 110; 1936, p. 57) characterizes the movement of *Synechococcus* as slow and irregular; in the smaller species, which are more bacteria-like in appearance, it is said to be barely distinguishable from Brownian movement. It is not altogether certain whether *Synechococcus elongatus* Naeg. with rod-shaped, narrow cells (cf. Geitler, 1936, p. 20, fig. 11, a–c) differs from *Pelogloea* Lauterb., and whether the two species of the latter (Lauterborn, 1915), supposed to be Green Bacteria (cf. p. 80), are not mere modifications.

In order to obtain young vigorous cells these small forms were grown in soil-water cultures (Pringsheim, 1946b). Slow, swinging movements were observed in *Synechococcus elongatus* Naeg., *Chroococcus turgidus* (Kütz.) Naeg. var. *nigricans* n.var., *Chroococcus sabulosus* (Menegh.) Hansg., and *Aphanocapsa rivularis* (Carn.) Rabenh., their rapidity depending to a large extent on the condition of the cells. When multiplication ceases the movements slow down considerably and may cease to be observable.

It remains doubtful if there are completely immotile Myxophyceae. Gliding stages seem to occur widely in multicellular genera, where they play a role somewhat parallel to that of swarmers of other algae. Unicellular propagation stages were called planococci by Borzi (1914), who observed their slow irregular movements, in a number of hormogonales. In a similar way unicellular stages of Rhodophyceae exhibit gliding movements, for instance *Porphyridium cruentum* (Vischer, 1935; Pringsheim, 1949a) and spores and spermatia of other species (Fritsch, 1945, pp. 429, 433, 597, etc.).

Of colonial Myxophyceae, *Merismopedia* (Niklitschek, 1934, p. 212) and *Holopedia* (Lund, 1942) are known to show active movement. The entire, tabular or foliose, colonies, when buried in mud, creep slowly out of it or move towards a better illuminated region. This can be inferred from observations in cultures; when their position is altered by turning the tubes through an angle the colonies move towards brighter regions. *Holopedia* readily dissolves into pairs of slightly curved rod-shaped cells which move still more quickly, so that their transloca-

tion can easily be observed. The movement is an interrupted one, with a tendency for attachment by one end, while the other swings round, i.e., similar to that described by Geitler (1925, p. 110; 1935, p. 57). It is characteristic that the movement appears to be undirected over short distances. Whether there is really only dissipation or perhaps also phototaxis, as in *Porphyridium*, remains to be decided.

The same kind of gliding movement was observed in pairs of spherical cells belonging to *Aphanocapsa*, as well as in *Tetrachloris inconstans* Pascher (Geitler and Pascher, 1925, p. 456), the nearest approach to a 'green bacterium' which I could examine. The cells of this minute form, 1.2 to 1.5 μ in diameter and arranged in diplococcus-like pairs, have a faint greenish tinge while larger groups appear bluish-green. Their shape and behavior resembles that of *Aphanocapsa*, but the movement of *Tetrachloris* is more lively.

Other Myxophyceae also exhibit phenomena which can scarcely be explained without assuming active locomotion. How can the social habit of epiphytic genera like *Dermocarpa*, *Clastridium*, and *Chamaesiphon*,[3] the branched colonies of the last and the net-shaped aggregates of *Pelodictyon* be understood without the assumption of motility? In *Chamaesiphon incrustans* (?) growing epiphytically on *Rhizoclonium hieroglyphicum*, the characteristic myxophycean movement could actually be observed, in detached vegetative cells as well as in the spores.

Gliding movements thus occur, not only in the Oscillatoriaceae and in the hormogonia of all the Hormogonales, but also in many Chroococcaceae, in Chamaesiphonales and Cyanochloridinae. Although Geitler (1936, p. 57) is of the opinion that the movement of unicellular stages is different in its nature from that of filamentous ones, there is in view of the existence of unicellular and multicellular hormogonia some possibility of its being essentially the same. There is thus little probability that colorless Myxophyceae would be indistinguishable from Bacteria, although a few debatable cases may still perhaps be found. Colorless Bacteria, parallel to the Chroococcaceae, like *Micrococcus*, *Sarcina*, etc. are different by their gram-positivity, and the gram-negative bacteria *Gonococcus* and *Meningococcus* are also not likely to be apochlorotic members of Myxophyceae, while true colorless members of the latter have been discovered and recognized as such by their gliding movements (see following chapter).

III. COLORLESS MYXOPHYCEAE

1. Chamaesiphonaceae and Oscillatoriaceae. The few colorless Myxophyceae so far known belong to all three of the main series, into which Geitler (1925) divides the class: Chroococceae, Chamaesiphoneae and Hormogoneae, or to three of Fritsch's five orders, viz. the Chroococcales, Chamaesiphonales and Nostocales. The colorless Chroococcales will be described in section (4) below. It is as yet not possible strictly to follow the classification of Myxophyceae when dealing with colorless forms.

[3] ***Ankistrodesmus*** **and** ***Ecballocystis*****, it is true, may have a similar habit.**

A pigment-free *Chamaesiphon*, described by Scherffel (1907; cf also Geitler, 1925, p. 151) is somewhat dubious. According to Geitler (1925, p. 148; 1932, p. 428) Scherffel's *C. hyalinum*, 'which may not be a blue-green alga at all', is faintly colored like *C. macer*, the bluish-green tinge of which is only recognizable in dense aggregates. Such very faintly pigmented species are not uncommon among Myxophyceae. Bavendamm (1924, p. 45) mentions another "colorless *Chamaesiphon*" without giving any description of it.

Among Oscillatoriaceae there are several forms with so little pigment that individual filaments may appear to be colorless. The minimum amount of chlorophyll sufficient to support adequate photosynthesis is not known, so that it is not justifiable to conclude that such forms are heterotrophic or mixotrophic. Moreover, Myxophyceae tend to bleach when the medium becomes deficient (Magnus and Schindler, 1912; Pringsheim, 1913; Boresch, 1913). *Pseudanabaena catenata* Lauterb. and *Oscillatoria formosa* Bory, for instance, look almost colorless in old exhausted cultures, although the trichomes are still motile. They soon recover their blue-green tint and multiply again when nutrient salts are added. Lauterborn (1915, p. 435) and Geitler (1925, p. 38; 1936, p. 8) record pale yellowish Myxophyceae which inhabit mud rich in hydrogen sulfide and which they regard as constituting a special ecological group. *Pseudanabaena catenata* and *Oscillatoria formosa* are supposed to belong to this group (Lauterborn, l. c., p. 437; Geitler, 1925, p. 372). As they are blue-green when in a healthy state, it is possible that the others would also turn blue when supplied with proper nutrients.

Species with a very pale bluish color are *Lyngbya endophytica* Elenkin and Hollerbach (Geitler, 1925, p. 402) inhabiting algal mucilage, and *Oscillatoria decolorata* West, stated to lead a saprophytic existence in stagnant ditches (Bhâradwâja, 1940, p. 204). *O. beggiatoiformis* (Grun.) Gom. is not colorless like *Beggiatoa*, but faintly blue-green. *O. angusta* Koppe (1924), again from oxygen-free mud (gyttja) is described as really colorless; but Geitler (1925, p. 365) questions the absence of pigments. Almost colorless is also *O. profunda* Kirchner from the bottom of a deep lake, and likewise *O. koprophila* Skuja (1948, p. 46) from a similar habitat. *O. beggiatoides* Arzichowsky (1902; cf also Kolkwitz, 1909, p. 153), which is described as colorless, and stores sulfur, is supposed to be a link between *Beggiatoa* and *Oscillatoria*. How it differs from *Beggiatoa* could not be ascertained as the paper was not accessible (cf. Bavendamm, 1924, p. 104).

In none of the instances of almost pigment-free species of *Oscillatoria* is the issue quite clear, owing to the lack of culture experiments. There is, however, no doubt as to the existence of chlorophyll-free Myxophyceae. *Beggiatoa*, when it lives saprophytically, is barely distinguishable from an apochlorotic *Oscillatoria* (cf. section *2*, below), while *Spirulina albida* Kolkwitz (1909, p. 137), which, like other Spirulinas, differs only in its spirally shaped body, is certainly chlorophyll-free and saprotrophic. Geitler (1925, p. 346) at first regarded *S. albida* as a very dubious form, but finding it later (1932, p. 927) on slides exposed in a stream, accepted it as a true heterotrophic member of Myxophyceae

(so also 1942, p. 34). The trichomes were colorless and showed not the slightest tint even when massed. In a recent letter he again expresses doubts regarding its taxonomic position. Apochlorotic Myxophyceae are not given much prominence in Geitler's books, and he tends to deny their existence.

Spirulina albida is not rare in England, occurring in the surface film of waters where the bottom is covered with black mud. It was twice cultured as a unialgal strain for nearly a year in a mixture of soil with a little organic matter but, although it was often subcultured, I was not successful in growing it free from bacteria. This was achieved by Dyar (1947) who found that on agar with sterile blood it multiplies and glides over the surface, freeing itself from contaminating organisms so that pure cultures were obtained. Dyar calls the organism *Spirochaeta plicatilis* Ehrbg., but her description and figures show clearly that it is *Spirulina albida*, and by her courtesy I was able to establish its complete identity with the material studied earlier.

The relation of *Spirulina* to *Spirochaeta*, which has repeatedly been discussed will be dealt with later (cf. p. 82). Here it suffices to mention that *Spirulina albida* is 0.8 to 1.2 μ wide, successive coils being 3 to 6 μ apart, and 2 μ wide, while *Spirochaeta plicatilis* is about 0.3 μ wide, the coils being close together and about 1 μ wide. *Spirochaeta plicatilis* is unicellular, while *Spirulina albida*, like other Oscillatoriaceae, consists of a number of cells, into which it disintegrates when injured.

The kind of movement is also different. *Spirulina albida*, like other members of its genus, has a certain degree of rigidity, although it bends slowly and slightly to and fro when entangled between detritus, as is often the case in its natural habitat. When mounted on a slide it commences after some time to glide in an almost straight line over the glass surface, meanwhile rotating on its axis. *Spirochaeta plicatilis* also rotates during movement, but does not keep a straight line, turning in various directions without apparent cause (cf. also p. 83).

2. Beggiatoaceae. When Bhâradwâja (1940, p. 204), in his review of the relations between Myxophyceae and thread-bacteria, states that 'heterotrophic colorless forms in the blue-green algae are not known to occur with certainty', he does not take the Beggiatoaceae into consideration because he believes their cell structure to be fundamentally different from that of the Oscillatoriaceae (l.c., p. 205). This opinion cannot be accepted (cf. p. 54).

The presence of sulphur droplets in the cells of *Beggiatoa* and Winogradsky's (1887) demonstration, more precisely confirmed by Keil (1912), that *Beggiatoa* can live without organic substances, have produced such an impression on biologists that the 'sulphur bacteria' were regarded as falling outside the range of other colorless organisms. This group was henceforth grouped together with others likewise oxidizing hydrogen sulfide and storing the sulfur inside their cells, like *Thiothrix*, *Thioploca*, many purple bacteria and certain colorless unicellular organisms (*Achromatium*, *Thiospirillum*, *Thiovulum*, etc. as Thiobacteriales (Bergey, 1926, p. 402)). The latter are, however, a conglomeration of taxonomically unrelated forms, and the discovery of sulfur also in pigmented Myxophyceae (Hinze, 1903b; Nakamura, 1937) shows that the sulfur organisms are a biochemi-

cal group like the iron organisms (Pringsheim, 1946b) and not a taxonomical unit.

Beggiatoa was in fact long ago recognized as a pigment-free relative of *Oscillatoria*. Vaucher described it under the name *Oscillatoria alba* as long ago as 1803. Cohn (1871/72, p. 186) included *Beggiatoa* and *Leptothrix* (syn. *Thiothrix*) as colorless subgenera of *Oscillatoria*. Van Tieghem (1880) states that certain Oscillatoriaceae (e.g., *Beggiatoa* and *Thiothrix*) lack chlorophyll. The name 'Sulphur Bacteria' (Winogradsky 1887, 1888) created confusion, although Solms-Laubach (1901) criticizes Wettstein for including *Beggiatoa* among the bacteria in opposition to the view then generally accepted.

It is, however necessary to consider whether the striking similarity is merely superficial and the result of convergent evolution, or whether a true relationship can be proved by other resemblances.

The little there is of differentiation in *Oscillatoria* is evident also in *Beggiatoa*. It is provided by the biconcave cells and the different end-cells of the filaments. Only the calyptra covering the tip in many species of the former genus is lacking in the latter. According to Geitler (1925, p. 338), 'The Oscillatoriaceae are probably not as little specialized as they seem. Their organization is complicated by segmentation, rhythmical growth and spiral symmetry of the cells.' The trichomes are slightly constricted at intervals, and the segments thus defined are of the same length as actively moving hormogonia. Each segment probably contains the descendants of a single cell (Geitler, 1932, p. 907, and own observations; cf. also *Caryophanon* p. 74 for comparison).

As observed in several species when actively growing, cell-division is not evenly distributed over the length of an Oscillatoria-trichome. Regions with short, evidently young, dividing cells alternate with others in which the cells enlarge but do not divide. The maximum length of the cells is reached only after several divisions, mostly the third (Geitler, 1932, p. 231). Owing to these morphological complexities and the great variety of forms Geitler (1925, p. 349) apparently believes that *Oscillatoria* is a relatively young and highly derived genus (although on page 36 of the same book he ascribes a great age to it), while 'the Beggiatoaceae by their simple cell-organization and their autotrophic nutrition belong to the most primitive plants' (Geitler, 1934, p. 191).

Actually it seems that the trichome organization is the same in *Beggiatoa* as in *Oscillatoria*. According to Hinze (1901, p. 373) 'Cell-division is markedly intercalary in *Beggiatoa mirabilis*; . . . in a relatively long filament, for instance, nearly a quarter of the cells are dividing almost simultaneously. Usually several adjacent cells, sometimes two, oftener three, divide at the same or nearly at the same time; during this period neighboring cells elongate and divide soon afterward . .' . The analogy is perfect and, as my own observations show, the smaller species of *Beggiatoa* behave in the same way as *B. mirabilis*. There is also in *Beggiatoa* a wide range of closely related forms, differing essentially only in their dimensions so that this genus might likewise be regarded as being in full evolutionary development, while the faculty of living chemo-autotrophically has nothing to do with primitivity.

The movements have been studied in both genera by competent investigators without producing a better explanation of their mechanism than has been obtained for other gliding organisms. The name *Oscillatoria* refers to the oscillating movements of the terminal part of the filaments. The oscillating appearance under the microscope is due to the optical projection of a conical circulation complicated by the friction between slide and slip which forces the tip to describe a narrow ellipse instead of a circle. It is the same in *Beggiatoa*.

Oscillatoria exhibits translocatory movements only when some part of the trichome is in contact with a solid body or the air-water surface. Forward movement is generally accompanied by rotation, and there is a certain relation between the two (Correns, 1897, p. 141), although some change of place can occur without rotation which seems to be lacking in certain species (Hansgirg, 1883) and in hormogonia (Harder, 1918). I have observed a faintly blue *Oscillatoria* and a narrow *Beggiatoa* moving to and fro without alteration of the slight, irregular undulation of the filaments and without rotation. The delicate mucilage sheath (Niklitschek, 1934) becomes visible in material mounted in fine suspensions (Correns, 1897, p. 146) and on agar, where it can be stained with safranine (Pringsheim, 1913, p. 67). The surrounding mucilage supposed to be lacking in *Beggiatoa* has been detected by Keil (1912, p. 362), and this removes the last morphological difference. In all these respects there is therefore complete agreement between *Oscillatoria* and *Beggiatoa* (cf. Kolkwitz, 1897, p. 463/64; and also own observations).

The trichomes both of *Oscillatoria* and *Beggiatoa* possess a certain degree of elasticity and straighten after having been bent. On the other hand they are also pliable, circumventing obstacles with which they come in contact during forward gliding. There is no spontaneous flexibility, bending resulting from resistance and disappearing with it (Kolkwitz, 1897, p. 461).

Small particles adhering to the filaments of *Oscillatoria* travel along the surface, at some places in one and at other places in the opposite direction. They thus form dense accumulations at certain points on the filaments. These accumulations are not static but change their positions. The direction of movement of the particles may reverse in any part of the filament (Correns, 1897, p. 144). The same can be said of *Beggiatoa* (Keil, 1912; and own observations). The phenomenon just discussed is no doubt in some way related to the still unsolved problem of the mechanism of movement.

In *Oscillatoria* rhythmic waves of contraction are commonly accepted as the chief cause of locomotion, as first suggested by Engelmann (1879, p. 55) and more clearly by Schmid (1923, p. 383). For *Beggiatoa* a similar view has been propounded by Benecke (1912, p. 150), and Ullrich (1926, 1929) demonstrated the existence of such waves in *B. mirabilis*. Other hypotheses that have been brought forward (cf. Fritsch, 1945, p. 802) are not very helpful. Even the contractile wave theory does not satisfactorily explain all the phenomena. Periodic contractions cannot well be the cause of the gliding movements of unicellular Myxophyceae (cf. p. 61), nor of those of few-celled or one-celled hormogonia, which Geitler (1935, p. 57) believes to be of a different nature. Contractile

waves have not at present been demonstrated in hormogonia at all (Fritsch, 1945, p. 803). Nor does the theory explain the role of mucilage-secretion which seems to be closely related to some still unknown mechanism concerned in the gliding of these organisms. Such a connection is suggested by the same phenomenon occurring in other gliding organisms. Myxobacteria (Jahn, 1924, p. 33), *Porphyridium* (Vischer, 1935, pp. 79/80), *Vitreoscilla* (cf. p. 71), as well as diatoms and amebas (e.g., *Naegleria Gruberi*; own observations) leave similar muclilage-tracks behind them, when moving over an agar surface. The importance of such residua and the contact with the substratum obtains support through observations that the direction of movement of gliding organisms is influenced by slight tensions in the agar (Stanier, 1942b, 1947b), as well as by their own previous mucilage-exudations.

It remains to compare the method of reproduction. In *Beggiatoa* and in numerous species of *Oscillatoria* the trichomes can grow to great lengths. Under unknown circumstances they break up into shorter units, called hormogonia, which exhibit lively movements and, by cell division and extension, again form long filaments. Their length is not related to the commencement of hormogonium formation. An improvement in nutritive conditions, after an interruption in development caused by a deficiency, seems to induce the onset of the reproduction phase.

Hormogonia are produced by active changes in the trichomes and develop specially shaped end-cells which artificially formed fragments are unable to produce. The injury resulting from mechanical fragmentation is often fatal, and short pieces of trichomes cannot be used for starting cultures. Even long filaments do not generally survive local lesions (Omelianski, 1904, p. 228; Cataldi, 1941). This indicates an intimate interrelation between the cells, such as is lacking in most other filamentous algae. The thread of an *Oscillatoria* or other similar member of Nostocales can be interpreted as an imperfectly septate derivative of a unicellular individual (Fritsch, 1942, p. 141; cf. also Crow, 1922, p. 85). The regions of dividing and enlarging cells, which can be distinguished in the trichomes, seem to be derived from one original cell. These are also the filamentous units which are transformed into hormogonia.

Beggiatoa again behaves like *Oscillatoria*. The filaments divide into portions which have the value of hormogonia and move actively (Winogradsky, 1888; Benecke, 1912, p. 205).

The great variation in width of the threads of *Beggiatoa* (Winogradsky, 1888; Klas, 1937) is remarkable. Winogradsky (l.c., p. 610) maintains that the species, which differ only in the diameter of the cells, are perfectly constant, but there is considerable variability in clone cultures. The definition of species in *Beggiatoa* is therefore still dubious (Bavendamm, 1924, p. 106). The situation is similar in certain species of *Oscillatoria*. In *O. princeps*, for instance, the variation in dimensions is far greater than one would expect in a well-defined species.

While there is thus striking resemblance between *Beggiatoa* and certain Oscillatorias, except in pigmentation and sulfur content, some authorities lay great stress on differences in the structure of the protoplast (cf. p. 54). Fischer

(1897) and Guilliermond (1926a) failed to find in *Beggiatoa* the differentiation into outer and inner protoplasm seen in many pigmented Myxophyceae, in spite of the employment of various staining methods. Guilliermond, who regards the central body as a primitive nucleus, says in regard to *Beggiatoa* (l.c., p. 581): 'on ne retrouve donc rien analogue à un noyau, ni au corps central des Cyanophycées.' Although an analogous conclusion of Bütschli's (1896, 1902) had already been disproved by Fischer (1897, p. 8), Guilliermond (l.c., p. 580) decides that the cytological structure of *Beggiatoa*, despite its general similarity to *Oscillatoria*, shows that it belongs to the Bacteria. Migula (1900, p. 40) states that '*Beggiatoa* follows *Oscillatoria* closely, even in its internal structure (existence of central body), so that the two genera can scarcely be separated', without giving the authority for his statement. Guilliermond's results have been confirmed by Petter (1933) and Delaporte (1939, p. 764); and Fritsch (1945, p. 860) summarizes the position in the words, 'The facts above detailed suggest the possibility that these types belong to a plurality of evolutionary lines diverging from a remote ancestry, from which the Myxophyceae also arose.'

In my opinion the loss of pigments may have been accompanied by a loss of differentiation within the cytoplasm (cf. p. 54), so that such a difference between *Beggiatoa* and *Oscillatoria* is not irreconcilable with their affinity. Skuja (1948 p. 46; cf. p. 62) has, however, observed a colorless species, referred by him to the latter genus, in which the central body is recognizable.

Thioploca is similar to *Beggiatoa*, except that the filaments are aggregated to form bundles enclosed within a common sheath. The analogy to *Hydrocoleus* and *Microcoleus* among pigmented Myxophyceae is obvious. The hormogonia of *Thioploca* enlarge into trichomes within the sheath without much movement. Lauterborn (1907), who discovered the genus, found the trichomes full of sulfur droplets and showing a bluish tint. Wislouch (1912) described a second species, having a faint bluish-green color, but only when the sulfur content was high. This does not suggest that the color is due to pigmentation as in the Myxophyceae, but rather that it might be due to some optical effect. Kolkwitz (1912), who was unable to detect any color in an otherwise identical organism, reports that 'the trichomes were as colorless as those of *Beggiatoa alba* present in the same preparation.' Both also presented the same appearance in dark-ground illumination, without any sign of fluorescence.

3. Thiotrichaceae. Thiothrix is customarily placed close to *Beggiatoa*, with which it shares the mode of nutrition and the storage of sulfur droplets in the cells (Winogradsky, 1888; Keil, 1912). It inhabits running water, where *Beggiatoa* would be washed away, being attached to a solid substratum by a basal holdfast. The absence of motility and the differentiation between base and apex, which is lacking in *Beggiatoa*, would in certain groups be regarded as sufficient for the establishment of a separate family. *Thiothrix* is therefore classed here as a representative of the Thiotrichaceae.

Reproduction is brought about by the detachment from the free ends of the trichomes of short lengths which give rise to new growths after attachment to some substratum. Winogradsky (1888) and Wille (1902, p. 259) describe

gliding movement of these reproductive bodies, but the formation of true hormogonia could not be confirmed by more recent observations. Keil (1912, p. 363) observed plenty of these hormogonia-like structures, but failed to find active movement, and I was equally unsuccessful. The capacity for gliding movement is, however, easily disturbed by changed conditions, and the settlement of young filaments is scarcely explicable without motile reproductive stages. True hormogonia may therefore well occur in *Thiothrix*.

If so, its nearest relatives must be sought among the pigmented Myxophyceae. Migula (1900, p. 40) does not include *Thiothrix* in the Beggiatoaceae, but in the Chlamydobacteriaceae; but the similarity between *Thiothrix* and *Leptothrix*, which ranked for some time as synonyms, is only superficial since the latter possesses flagellate reproductive stages (cf. p. 78). No blue-green alga, parallel to *Thiothrix*, seems to be known. The nearest approach is constituted by attached species of *Lyngbya*. The formation of few- or one-celled hormogonia from the ends of the filaments does not seem to have been observed in this genus, but it occurs in other Myxophyceae.

4. Achromatiaceae. The forms included in this family, the type of which is *Achromatium oxaliferum* Schewiakoff, are unicellular, deposit sulfur droplets and exhibit a peculiar movement. There are a number of genera: *Achromatium* Schewiakoff 1893, *Modderula* Frenzel 1897, *Hillhousia* West and Griffiths 1909, 1913, *Thiophysa* Hinze 1903, and Nadson 1914. Some of these names, perhaps even all, seem to be synonyms. *Achromatium* may, however, represent the freshwater and *Thiophysa* the salt water species. *Thiovulum* and *Thiosphaerella*, sometimes placed close to *Achromatium*, are flagellate and do not therefore belong to the family.

The name Achromatiaceae seems to have been used first by Buchanan 1918 (Bergey's Manual, 1926, p. 402) for an assemblage of unrelated forms, including for instance *Thiospira* Wislouch 1914 which is morphologically a typical flagellate *Spirillum*. The same confusion is found in Buchanan's contribution to Bergey's Manual of 1939 (p. 910). Moreover, the author maintains that 'Cell division (in *Achromatium*) resembles the constriction of flagellates rather than the fission characteristic of bacteria', forgetting that in the Coccaceae cell division is generally of this type.

As a more natural classification, the family Achromatiaceae is here restricted to non-flagellate organisms showing gliding movements, and constitutes the unicellular parallel to the Beggiatoaceae. The first organism of this group to be observed was interpreted as a stage in the life cycle of *Beggiatoa mirabilis* (Cohn, 1867), with which it was associated. This form was marine, and Kolkwitz (1909) refers it to *Thiophysa*.

The practice of certain authors to regard flagellate, and non-flagellate, gliding species as near related, or even as belonging to the same genus, must be abandoned. Ellis (1932), for instance, at one place (p. 116) speaks of cilia in *Thiovolum* and at another place of its being devoid of cilia (p. 125), while the authors of *Hillhousia* erroneously describe it as having peritrichous flagella, whereas sulfur bacteria have polar flagella, if at all; this latter mistake is repeated by Buchanan

(in Bergey, 1939, p. 910), although the character of the movement could not be explained on this basis. Lauterborn's (1915, p. 413) *Achromatium mobile* (syn. *Macromonas* Utermöhl and Koppe; *Microspira* Gicklhorn) is described as possessing a polar flagellum but, although it was found in company with *Achromatium oxaliferum*, it cannot be grouped in the same genus with this gliding organism which has no flagella.

In *Achromatium* there is a considerable range of sizes. Bersa (1920, p. 233) found the length to be between 9 and 75 μ, with all intermediate lengths represented, the usual dimensions being 30 to 40 μ long and 10 to 18 μ broad. The cells of Nadson's (Ellis, 1932; Nadson and Wislouch, 1923?) *Achromatium gigas* even reach a length of 102 μ, while Bavendamm (1924) records small individuals, as short as 3 μ. The occurrence of forms showing a wide range of widths is one more parallel to *Beggiatoa*.

Achromatium, in its movements and morphological features, resembles *Synechococcus* in the family Chroococcaceae among Myxophyceae. Stanier and van Niel (1941, p. 453/54) rightly say: 'it is tempting to compare them (i.e., *Achromatium*) with such members of the Myxophyta as *Synechococcus aeruginosus*, to which they might well be related in the same manner as *Beggiatoa* is to *Oscillatoria*.' Both forms are cylindrical with rounded ends and divide by median constriction. *Synechococcus aeruginosus* is the largest species, being about 60 μ long and 40 μ wide. The habitats occupied by the two genera are, however, very different, so that a direct transformation of *Synechococcus* into *Achromatium* cannot have taken place.

The movement of *Achromatium* has been described by Schewiakoff (1893), Zacharias (1903a), and Virieux (1912, 1913) as spasmodic, slow, rolling, changing its direction irregularly, jerky, interrupted,—in short a very different kind of movement from that of flagellate bacteria, indicating that it does not belong to the Bacteria, to which it was so often relegated. The movements of *Synechococcus* are described in the same way, almost with the same words (cf. Geitler, 1925, p. 110; 1936, p. 57). According to Virieux (1913, p. 267; West and Griffiths 1913, p. 83) and my own observations *Achromatium* adheres to the slide by a thin mucilaginous exudation, and the same has been observed by me in *Synechococcus elongatus* n. var. *parva*.

Virieux (1912, p. 279/80) records that, in addition to division of cells into two, the formation of large numbers of endospores takes place in *Achromatium*. This method of spore production is known in many Myxophyceae, for instance in *Aphanothece*, a genus similar to *Synechococcus* (cf. Fritsch, 1945, p. 810), but not in any member of the Bacteria.

Thiophysa, with the marine or brackish-water species *T. volutans* Hinze and *T. macrophysa* Nadson, appears to be related to *Achromatium*, although it is difficult to assess this on the little information available. It is still more difficult to say anything definite about *Thiovulum Mülleri* Warming (syn. *Achromatium Mülleri* (Warming) Migula (cf. Molisch, 1912, p. 55)) and *Thiosphaerella amylifera* Nadson, which Ellis (1932, p. 116) groups among the Achromatiaceae. The ellipsoid cells of *Thiovulum majus* (Hinze, 1913, p. 198) are stated to be covered with

flagella 0.7 μ thick and to exhibit a movement which is more rapid than that of *Achromatium*. The 'flagella' appear to be bacteria like those described in *Hillhousia* by West and Griffiths (1913, p. 83).

5. Vitreoscillaceae. As stated above, *Beggiatoa alba* can live heterotrophically without depositing sulfur and cannot then be distinguished from a colorless *Oscillatoria*, although the existence of such species is not established beyond doubt. Geitler, in his various synopses, says little about pigment-free members of Myxophyceae, and other authors do not mention them at all, whereas, according to Fritsch (1945, p. 871) 'there can be no doubt that colourless Myxophyceae occur in a free state in nature'.

There are, in fact, numerous colorless, filamentous, gliding organisms, which are by no means rare and are easily demonstrated, so that it is difficult to understand how they have escaped notice. They live in a variety of habitats and are almost regularly present amidst floating masses of filamentous blue-green algae; other species live in cow dung, in the mucilage of various organisms (e.g., *Ophrydium*, accompanied by *Oscillatoria amphibia*), and between débris in the surface scum of stagnant waters, particularly those with a bottom of black mud. Seven species, one marine, have so far been isolated in pure culture, although in many cases attempts to culture such organisms have failed. While all have distinctive features of their own, some are similar to a colorless *Oscillatoria* or a sulfur-free *Beggiatoa*, so that they were suspected of belonging to the latter genus until the inability to deposit sulfur from hydrogen sulfide had been established.

It is proposed to include these organisms in the new family Vitreoscillaceae, with *Vitreoscilla beggiatoides* as the type. Four of the species, that have been cultivated possess cylindrical threads, while the others are more or less constricted between the cells so that the trichomes are moniliform. Most of the species tend to break up into short lengths (hormogonia) more readily than *Beggiatoa alba*, and only in one of them do the filaments reach a great length under favorable circumstances.

The length of the trichomes and the degree of constriction are so variable, even in one and the same strain, that distinct genera cannot be established, although some of the forms not yet grown in culture differ more profoundly. The various species seem to be confined to certain habitats, but the chief characters are furnished by the width and length of the cells, the tendency to fragment into short lengths, the rapidity of movement and the cultural behavior and appearance. Other differentiating features may be found by more detailed physiological and cytological investigations.

The cells have a low refractive index, rather like that of Myxobacteria, so that the small, narrow species are not readily observed under low power. The cells are gram negative, but stain well with bacterial dyes and vital stains. Old or unhealthy growths contain fat and volutin, while those in vigorous development appear optically almost empty. They possess nucleoid structures not very different, as far as investigated, from those of Bacteria. Cell-division takes place by diaphragm-like ingrowth of the cell wall, much as in Myxophyceae and Bacteria. Hormogonia are initiated by certain constrictions deepening to form circular grooves, so that the descendants by division from one original cell are

eventually separated from others. The frequency of such fragmentation varies within the same species with the conditions of growth, as does also the length of the cells.

These organisms, which earlier biologists would probably have regarded as Bacteria, differ from them essentially in their gliding movements. These also facilitate their separation from other microörganisms during the preparation of pure cultures. The trichomes of *Vitreoscilla* escape from aggregations of other organisms which cannot move over an agar surface, so that the technique of their isolation is the same as that devised by Cataldi (1940) for *Beggiatoa*. *Vitreoscilla beggiatoides*, the first species grown in pure culture, was, however, purified by washing by the pipetting method.

Four species were isolated from cow-droppings on plates of dung-agar, but the pure cultures grew better on other media. The remaining 3 species were isolated using an agar medium with neutralized yeast extract. Relatively low concentrations of proteid substances, ranging from 0.05 to 0.3% were found to be most suitable, not only to obtain pure cultures, but also to maintain them in a healthy state. None of these organisms seems to live in heavily polluted water. Although not specially delicate, they do not stand competition very well.

The gliding habit determines the nature of the growths on agar, most species producing wavy, curly, or spiral colonies on media of not too high a nutritive value. On rich media drop-like colonies, resembling those of many bacteria, are formed. In the former case single filaments project and start growths on free portions of the agar surface. The projecting strands gradually become wider as the trichomes elongate and glide past one another, forming outgrowths and twirls. A characteristic feature is the production of new colonies, at a distance from the original growth, from individual trichomes which leave tracks behind them as they glide forward. Such secondary colonies cannot be formed by organisms lacking the capacity for gliding movement, although the growths may otherwise be strikingly similar owing to the possession of long threads by the organisms concerned. This is so, for instance, in *Bacillus mycoides*, *Lineola longa* (Pringsheim and Robinow, 1947), and *Sphaerotilus natans* (Pringsheim, 1949a), three bacteria which, though not closely related, all produce spirals and curls but show always a connection with the place of inoculation.

All the species of *Vitreoscilla* resemble *Beggiatoa* in being able to live on peptone, beef extract, yeast extract and the like. Sugar produces no favorable effect, and that of acetate is not very pronounced. This group therefore seems, like the majority of heterotrophic microörganisms, to have no specific biochemical properties, but to utilize compounds formed in the bacterial decomposition of proteins. None of them seems to be able to hydrolyze genuine proteins. Casein is not dissolved, nor is gelatine liquefied.

In liquid media growth occurs only with concentrations of nutrient substances even lower than in agar media. A suitable solution contains 0.04% yeast extract, 0.04% Bacto tryptone, 0.02% beef extract, and 0.02% sodium acetate. Mixtures are on the whole better than single substances. A medium composed of 0.1% yeast extract and 0.1% Bacto tryptone needs the addition of soil extract, this mixture being the best so far tested. Multiplication is never as luxuriant as

on agar, but the trichomes appear healthier and more like the original material, and show pronounced motility, seen especially well at the margin of small hanging drops on a fat-free cover-slip.

Apart from the species grown in pure culture, only a few others presenting characteristic features have so far been distinguished. It is doubtful whether they should all be included in the genus *Vitreoscilla*. A few are described below.

The specific name of *Vitreoscilla flagellum* refers to the lively movements of its narrow trichomes (about 0.3 μ wide) which almost give the impression of a flagellum of *Euglena*. Cells could not be distinguished. The length of the trichomes varies greatly, the shortest not exceeding 10 μ, while the longest are so much curved and entangled that they cannot be measured, although certainly more than 100 μ long. This species was found frequently among filamentous Myxophyceae, such as *Oscillatoria amphibia*. It resembles *Vitreoscilla marina*, but is narrower and confined to fresh water.

V. stricta was found in a pond with water fowl and in a pool polluted by cattle, both containing iron organisms and a multitude of pigmented flagellates. This species is characterized by its rigid trichomes which during the lively gliding movements bend only when caught between obstacles and straighten out immediately when they become free. In the marked elasticity of the trichomes, which are about 1 μ wide, it differs from other species of the genus, as well from *Beggiatoa*, but only in degree. The cylindrical cells were full of small granules.

An organism found only as a few individuals, though in various places between debris and algae may be named *Bactoscilla flexibilis*. The generic name refers to the fact that the short trichomes are composed of a number of rods, about 0.5 μ wide, apparently separated by empty interspaces. The peculiar feature of this genus is the slow, though pronounced bending, during which the individual rods remain straight. The structure is therefore comparable to that of *Lineola articulata* n.sp. (unpublished); but while this filamentous bacterium swims with the help of peritrichous flagella, *Bactoscilla* is a gliding organism. In both forms, it is the invisible matter constituting the hinge-like joints that bends. This is similar, though far less evident, in other members of the Vitreoscillaceae, for instance in *Vitreoscilla moniliformis*, the widest species of all, as well as occasionally in *V. paludosa*, but, owing to the shortness of the cells, not quite certain. The occurrence of such a mechanism, although obscure in its physical nature, renders a general application of the contractile wave theory still more difficult.

Certain yet smaller filamentous forms, to be grouped in the same genera or in a third one, *Microscilla*, are only mentioned here to illustrate the range of unknown organisms still to be described. They may possibly have some relation to the Myxobacteria, to which, as filamentous members, they may form a transition.

IV. ORGANISMS WHOSE AFFINITY WITH BACTERIA AND MYXOPHYCEAE IS DISPUTED

1. Caryophanon and Oscillospira. *Caryophanon* was discovered and isolated by Peshkoff (1940). It is the largest bacterium ever grown in pure culture, and

for this and other reasons of considerable interest. Peshkoff's title 'Phylogenesis of two microbes *Caryophanon latum* and *C. tenue*, organisms which are intermediate between Blue-green Algae and Bacteria' explains why some space must be devoted to a discussion of these organisms.

Although most of the features stated to be characteristic of the genus could be corroborated by an investigation of *C. latum*, Peshkoff's cytological data were found to be incorrect, and his views as to taxonomic relations had to be refuted (Pringsheim and Robinow, 1947). It is curious that Peshkoff (l.c., p. 615/16) although admitting that *Caryophanon* is a 'typical bacterium . . . owing to its peritrichate flagella and some other features', believes that 'the type of its nucleal apparatus suggests its direct relationship to the Blue-green Algae.' In actual fact it is, just as in *Oscillospira*, the appearance of the trichomes which suggests a similarity to the Myxophyceae and not the nature of the cell-contents.

Peshkoff devoted much attention to the nuclear apparatus but, owing to an unmethodical use of various fixing and staining methods, he misapprehended the cellular and nuclear organization. As nuclei of *Caryophanon* he describes various structures which cannot well be identical, to wit (1) Denser regions in the living organism and arranged in a row within the filaments are interpreted as nuclei, hence the generic name. They are also shown in some of his photographs of stained preparations, where they appear as elongate structures placed transversally, or as V-, H-, and X-shaped bodies. They probably comprise the whole or nearly the whole of the protoplast, which is repeatedly cut into by penetrating cross-walls during the process of cell division. (2) In certain captions to the colored figures in the first publication (1940) rings are described as nuclear structures; these seem to be in part nucleoid bodies deformed by unsuitable preparation and in part developing cross-walls. (3) The beaded structures, lining the inner surface of the outer walls in one of the photographs, are again of a different nature, probably a cytoplasmic granulation in the ridges flanking inwardly progressing cross-walls. (4) There are indications that Peshkoff has also seen the true nucleoids which, as in other bacteria, are better demonstrated by Giemsa staining after hydrolysis (Pringsheim and Robinow, 1947). None of these structures much resembles the Feulgen-positive granules described in the few Myxophyceae so far investigated (Poljansky and Petruschewsky, 1929; cf. also p. 54).

Peshkoff is also wrong in ascribing a non-cellular organization to *Caryophanon*. It seems that the absence of cross-walls, the existence and mode of formation of which can be demonstrated in *Caryophanon* more readily than in most other bacteria, was suggested to him by earlier studies (1938) of the cell structure of a bacterium, obviously misshapen by 'involution'. It is not clear why the lack of cross-walls is regarded as a feature by which *Caryophanon* approaches the Myxophyceae, since the trichomes of the latter are always composed of numerous cells.

Peshkoff does not mention the peculiar morphological structure of *Caryophanon*, either in the Russian text or in the English summary, as a feature relating it to certain Myxophyceae, although chains of discoid cells like those constituting the body of *Caryophanon*, so common in Oscillatoriaceae, were not

known in any bacterium; moreover the breaking up of the filaments into short lengths is reminiscent of the hormogonia of Myxophyceae, while filamentous bacteria were believed to dissolve into single cells.

These rather striking resemblances do not, however, appear to be of taxonomic importance. The shortness of the cells of *Caryophanon* can be correlated with their extraordinary width (2.5 to 3.2 μ) and indicates a tendency opposed to the formation of over-large cells, a tendency which can also be recognized in *Oscillatoria*, if the shapes of cells in species with different widths are compared. As a rule the narrow species have elongate, the wide ones short cells, a rule already recognized by Gomont (Geitler, 1936, p. 25).

The hormogonia of Myxophyceae are short lengths of filament which are characterized by the capacity for movement (Fritsch, 1945, p. 804). Up to the time of Peshkoff's publications, such structures were unknown in Bacteria, and they differ of course in *Caryophanon* from those of Myxophyceae in that movement is effected by flagella. Another difference is afforded by the absence in *Caryophanon* of any indication of a life-cycle as there is in *Oscillatoria* where periods of vegetative growth alternate with such of hormogonia production. When growth is undisturbed in *Caryophanon*, the trichomes develop constrictions which appear at regular intervals and steadily deepen until that first formed and situated approximately at equal distances from the ends divides the trichome into two. No *Oscillatoria* seems to be provided with such marked constrictions indicating at an early stage the place of fragmentation; but the alternation in this genus between portions exhibiting cell-division and others exhibiting elongation shows that there is another kind of periodicity in *Oscillatoria*, the formation of hormogonia being initiated by external factors and commencing with the death of certain cells. This divergence in organization is best explained on the assumption that the similarities between *Caryophanon* and certain Myxophyceae are superficial and due to convergent evolution, rather than to a common origin, while the difference between *Caryophanon* and other bacteria has become less marked since we know that bacterial rods are often composed of several cells (Robinow, 1944). They then divide in essentially the same way as *Caryophanon* trichomes (cf. also Bisset, 1949).

Peshkoff's recognition of *Caryophanon* as a form intermediate between Bacteria and Myxophyceae cannot be accepted. There are no intermediate forms; the two classes are independent. Peshkoff (1940, p. 616) attempts 'to find out what bacteria-like organisms described in scientific literature could be classified as the nearest relations to *Caryophanon*.' In this connection he mentions as closely related *Oscillospira*, which is dealt with below, and *Pontothrix* (Molisch, 1912), a member of Chlamydobacteriaceae. The polar flagella on the swarmers of the latter (cf. p. 78) show that it is widely removed from the peritrichous bacteria *Caryophanon* and *Oscillospira*.

Organisms of the Oscillospira group have frequently been described, usually as colorless parasitic Myxophyceae. It is difficult to assess the taxonomic relationship between the forms observed by the various authors. They are: *Arthromitus intestinalis* Valentin (1836), *Entomitus batrachorum* Collin (1912/13), *Oscil-*

lospira Guilliermondi Chatton and Pérard (1912/13), *Oscillatoria caviae* Simons (1920), *Simonsiella Mülleri* Schmid (1922), *Anabaeniolum brumpti* Langeron (1923), *Anabaeniolum Langeroni* and *A. longum* Nadson and Krassilnikov (1928), *Oscillatoria cyprini* Léger and Bory (1932), and *Dactylococcopsis echini* Mortensen and Rosenvinge (1934). Grassé (1924) recognized three genera: *Oscillospira* Chatton and Pérard, *Entomitus* n.g. (syn. *Arthromitus* Collin) (?) and *Simonsiella* (Schmid), but there may be more synonyms. A relationship with the Myxophyceae was already assumed by Chatton and Pérard.

All members of the group inhabit the intestines of animals, and occur in a great variety of them, especially in the rumen of ruminants and the cecum of rodents, but some are found in batrachians and others in the human mouth. Morphologically they are characterized by the possession of filaments composed of a limited number of discoid cells with approximately hemispherical end cells, cell-divisions being accomplished by diaphragm-like ingrowth of the wall. In certain instances (e.g., *Oscillospira*, Delaporte, 1934) endospores have been found, but these seem to be rather erratic in their occurrence. Grassé (1924), who found them in all the members of the group investigated, believes they are universal. Some authors record motility, while others do not mention it or failed to observe movement. Reproduction is effected by fragmentation into short lengths, in a way similar to that in *Caryophanon*.

While Harder (1920), Pascher (1921), Schmid (1922), Canabaeus (1929) and others, who were well acquainted with Myxophyceae, follow Chatton and Pérard and Simons in regarding *Oscillospira* and its relatives as apochlorotic members of this class, Geitler (1925, 1936), Bhâradvâja (1940), and Fritsch (1945, p. 871) doubt their assignability to the Myxophyceae.

Pascher (1921) speaks of the group as parasitic Oscillatoriaceae and points to 'the quite analogous occurrence of colorless Oscillatoriaceae in the dead bodies of Rotatoria and Cladocera' which he repeatedly found filled with colorless filaments sometimes even occurring in the live animals. According to him almost or completely hyaline Myxophyceae also live in the mucus of other organisms (cf. pp. 62, 70). He constructs a (rather artificial) evolutionary series, commencing with normal blue-gree forms and passing through faintly pigmented to colorless Myxophyceae, as was already done by Simons (1920, p. 363). Pascher's views do not seem to be based on personal knowledge of *Oscillospira* or similar organisms, and there is no evidence that the transformation of chlorophyll-containing into apochlorotic organisms has ever gone this way.

Geitler (1925, p. 338; 1932, p. 909; 1936, p. 1) rejects the idea that these organisms are Myxophyceae. According to him they '. . . have a certain resemblance to *Oscillatoria* and have therefore been described as such. The occurrence of peculiar, large, refractive, not readily stainable structures, which are probably spores, and the quick movements observed in certain forms are against grouping them among the Myxophyceae. They are provisionally to be referred to the Bacteria.'

Actually there is little that speaks in favor of a close relationship between the Oscillospiraceae and the Myxophyceae, except a superficial resemblance in Oscil-

lospira to certain species of *Oscillatoria* with short discoid cells. As pointed out above, this characteristic is also found in *Caryophanon*, whose similarity to *Oscillospira* was already recognized by Peshkoff (1940, p. 616) and resulted in a similar error. There are in fact scarcely any pigmented Myxophyceae that resemble *Oscillospira*, the filaments of the former generally being much longer and lacking endospore-formation (cf. also Bhâradvâja 1940, p. 204), while the kind of movement is also different.

In his first paper Simons (1920, p. 361) described the movement of his *Oscillatoria caviae* as resembling that of *Oscillatoria* and *Beggiatoa*, but later (1922, p. 503) he points out that the speed of movement considerably surpasses that of any *Oscillatoria*, and this should have warned him as to the difference. I failed to observe motility in ordinarily mounted preparations from the rumen of sheep and the cecum of guinea pigs, although *Oscillospira* was often present in enormous numbers. The movement was therefore suspected to be affected by access of air. Robinow and I (1947, p. 274) observed motility only in sealed preparations where it was similar to that of *Caryophanon* in cow dung. This suggested the presence of flagella, which Robinow was able to demonstrate.

Mr. Frank Baker of the Rowett Research Institute, Bucksburn, Aberdeenshire (private communication; cf. also Baker, 1933, 1943) was kind enough, in response to my request, to test our observations on a rich material. By working with a warm stage and sealing the cover-glass with wax, he regularly obtained free swimming by healthy trichomes from the rumen of sheep and cattle and the cecum of guinea pigs. He informed me also of the occasional occurrence of spores in material from these sources. Mr. Baker believes that the Oscillospira-like organisms from ruminants and rodents are 'generically, if not specifically, identical. . . in view of: (1) the very similar internal organization of the filaments; (2) the formation of hormogones and (3) of spores; (4) the formation within the filaments of a substance giving a blue-mauve reaction with iodine.' The variation in width of the trichomes is probably due to the fact that they taper markedly towards one or both ends, so that hormogonia of different diameters are produced.

Oscillospira is therefore a bacterium, resembling *Caryophanon* in its short trichomes composed of wide discoid cells and provided with peritrichous flagella, but differing in (1) the still greater diameter of 3 to 7.5 μ, compared with 2.5 to 3.2 μ in the latter; (2) the anaerobiotic characteristics;[4] (3) the starch-like reserve substance 'granulose', resembling that of *Clostridium* and several small organisms associated with *Oscillospira*; (4) the spore formation. The ovoid spores are peculiar in that they are too large to be accommodated by single cells, so that several break down to for a spore chamber. The spores appear to be of the same type as in bacteria, not like the ordinary spores (akinetes) of Myxophyceae.

Although all the organisms of the Oscillospira-group have a similar appearance, their affinity is not in every case established. For instance one form recorded as frequently occurring in the human mouth (Miller, 1892; Fellinger, 1924) may

[4] There are, however, indications of aerobic growth on agar plates (Simons, 1922, p. 509; Hocquette, 1933), and of occurrence in the mouths of mammals (Simons, 1922, p. 508).

perhaps be different. Delaporte (1934) demonstrated a nucleal reaction in bodies situated in the center of the cells of several forms, but further cytological and cultural investigations are required to clarify the situation.

2. Chlamydobacteriaceae. The supposed relationship between the Chlamydobacteriaceae and certain Myxophyceae has a long history and is linked with the names of some of the best microbiologists. It is still supported at the present day, although there is little substance in it.

Cohn (1870–71, p. 118) compares the filaments of *Frenothrix* to colorless Oscillatoriaceae. Migula (1904–07, p. 129) believes that of the Bacteria the Chlamydobacteriaceae are nearest to the Myxophyceae. Kolkwitz (1909, p. 76) places colonial and filamentous Bacteria and Myxophyceae with a similar cell arrangement side by side. He compares *Sphaerotilus* and *Cladothrix* with *Scytonema*, *Clonothrix* with *Rivularia*, and *Crenothrix* with *Chamaesiphon*. None of these pairs of forms are really parallel in their morphological structures. Passing over the intermediate period, Bhâradwâja (1940, p. 203) may be mentioned as repeating similar arguments in more recent years. The resemblance between *Cladothrix* and *Crenothrix* and certain Myxophyceae is also mentioned by Fritsch (1945, p. 860) who, however, abstains from drawing rash conclusions.

The view of a similarity between Chlamydobacteriaceae and certain Myxophyceae is based on (1) the presence of sheaths around the trichomes; (2) the propagation by short lengths of filament; (3) the 'false' branching of *Cladothrix* which is also found in many Myxophyceae; (4) the erroneous belief that trichomes of *Leptothrix* creep out of their sheaths and form new ones. Such gliding movements could not be observed, either in microscopical preparations or on agar plates (Pringsheim, 1949a, p. 459); (5) the deposition of ferric and manganic compounds on the surface of certain Myxophyceae, as in the Chlamydobacteriaceae.

Owing not so much to such parallels, but merely because of the true filamentous habit, although this is found in many other bacteria, the Chlamydobacteriaceae have been coupled with the Beggiatoaceae as Trichobacteria or Desmobacteria in most systems of classification of bacteria (cf. p. 7). This practice is misleading and has been abandoned by Stanier and van Niel (1941, p. 455/56), although they maintain the suggestion that '*Clonothrix fusca* is probably a colorless blue-green alga whose counterpart might be found in the myxophytal genus *Rivularia*.' If the writers have in mind *Clonothrix fusca* Roze (1896), for which its author asserts a bluish tint and consequently describes it as a member of the Myxophyceae, then we are not dealing with a colorless organism. If, however, they are thinking of a form identical with or very similar to *Cladothrix*, such as Schorler's (1904) *Clonothrix fusca*, then there is scarcely much similarity with *Rivularia*. Regarding *Sphaerotilus*, *Leptothrix*, and *Crenothrix* Stanier and van Niel are more cautious. It is not clear, however, why they believe that 'the morphological characteristics of *Crenothrix* are so outstandingly different from those of the other two genera'. Although it seems that *Crenothrix* does not produce swarmers like *Sphaerotilus*, and although its conidia are formed by division along three planes instead of one, these are not very important differences. The reproductive stages of *Sphaerotilus* may also be non-motile, and longitudinal divisions may occur

(Pringsheim, 1949a). Moreover, non-motile aplanospores are produced under certain circumstances in place of swarmers by many algae.

There is, however, a blue-green alga which is really strikingly similar to *Crenothrix* but for the color: *Letestuinema Bourrellyi* (Hortobágyi, 1947). It shares with *Crenothrix* the lack of heterocysts and hormogonia and propagates with the help of spores which are produced within an apically widening sheath by transverse and longitudinal division. They round up before being discharged. Nothing about motility is known.

Even those characteristics of Chlamydobacteriaceae which most recall those of Myxophyceae are, in fact, on closer inspection rather unlike. (1) The sheaths of Chlamydobacteriaceae are very thin and tough, and the cells of older threads are often separated from one another. This is not so in any member of Myxophyceae; (2) while the hormogonia of the latter are well-defined portions of filaments originating by separation at prepared places, the Chlamydobacteriaceae reproduce by irregular fragmentation into short lengths. Moreover, the hormogonia of Myxophyceae exhibit gliding movements, while the reproductive cells or cell-aggregates of Chlamydobacteriaceae are either motionless or, in the possession of flagella, resemble swarming bacterial rods; (3) the false branching of *Cladothrix* is not so well defined as that of Rivulariaceae and Scytonemataceae. There are neither heterocysts, nor necridia. The branching of *Cladothrix* may be suppressed altogether when the sheaths are either too soft to hold the parts together or too rigid for cells to break through and to give origin to branches.

Too much weight may therefore not be given to the similarities which really all depend on one feature, the enclosure of the trichomes in sheaths which has developed by parallel but independent evolution in the two groups. The Chlamydobacteriaceae are true Bacteria, more nearly related to the Spirillaceae than, for instance, are *Corynebacterium* or *Mycobacterium*, or probably even *Bacillus*.

3. Photosynthetic Bacteria. There are many bacteria-like organisms of minute size which possess pigmented protoplasm and whose relation to true Bacteria and to Myxophyceae has some bearing on the problems discussed in this review. Their color is green, yellowish green, blue-green, brown, red, or purple. While the Purple Bacteria or Rhodobacteria and the brown Phaeobacteria are closely related (van Niel, 1944), those with a more or less green shade belong to various unrelated groups.

a. Green Bacteria. The designation 'Green Bacteria' has merely a descriptive value. The earlier records (Perty, 1852; van Tieghem, 1880; Engelmann, 1882) can scarcely be adjudged since diagnoses, measurements and figures are lacking. Dangeard (1895), and in part also Ewart (1897), possibly mistook small Chlorophyceae for green bacteria. Zopf (1882), Cohn (1897, p. 494) and Schmidle (1901) refer such organisms to the Algae. Benecke's (1912, p. 243) statement that blue-green Spirillas bearing flagella have been observed has not been substantiated. Baas-Becking (1925, p. 630) found a minute green coccus and a green streptococcus-like organism, which he believes to be identical with Ewart's *Streptococcus varians*. He succeeded in growing the latter on peptone agar, though not in pure culture; and he concludes that it is aerobic.

Green Bacteria were, however, for the most part found associated with organisms adapted to a high concentration of hydrogen sulfide (Szafer, 1911; Skene, 1914; van Niel, 1931), such as Purple Bacteria and Myxophyceae, or in mud and stagnant water rich in organic matter, or as symbionts on the surface or within the body of various small organisms.

Geitler and Pascher (1925, p. 451) published a fairly comprehensive treatment as an appendix to the Myxophyceae and proposed to change the older name of Chlorobacteriaceae (Lauterborn, 1915, p. 425) into Cyanochloridinae. According to them the bacterial nature of these organisms has not been proved in a single case. They admit that they represent a non-homogeneous assemblage, the majority of whose members are not blue-green but yellowish. This does not preclude an affinity with the Myxophyceae, which is highly probable in many cases.

Van Niel (1931, p. 73) subjects Pascher's (1914) and Geitler and Pascher's (1925) descriptions of numerous symbiontic and free-living Green Bacteria, as well as their grouping as Cyanochloridinae near to the Myxophyceae, to severe criticism. He himself found only one species which he isolated in pure culture and which he believes to be identical with *Chlorobium limicola* Nadson (1906, 1912), as well as with the greenish rods belonging to Lauterborn's (1915) *Chlorochromatium aggregatum*, and even with Pascher's *Cyanarcus hamiformis*, *Chroostipes linealis* and certain other Cyanochloridinae.

Although Pascher may sometimes have been rash in naming occasional discoveries, van Niel's distrust goes too far (Pringsheim, 1932a). He underestimates the importance of morphological investigations. Moreover several of these minute organisms do not possess the yellowish to olive-green hue of the green sulfur bacteria, but are blue-green like true Myxophyceae, as in Pascher's *Cyanarcus* and *Chroostipes* (Geitler and Pascher, 1925, p. 116). I myself observed the blue-green color in several bacteria-like organisms which I studied in cultures. How vague the description of color may be, is shown by Geitler and Pascher's (l.c., p. 460) diagnosis of a form similar to *Chlorochromatium* as 'gelbgrün mit einem ausgesprochenen Stich ins blaugrüne' (yellowish green with a distinctive blue-green tinge), a contradiction in itself.

The pigments of Green Bacteria have only been investigated by Metzner (1922), who discovered a new green pigment bacterioviridin, differing from chlorophylls a and b, as well as from the bacteriochlorophyll of Purple Bacteria. The pigments will probably be the most suitable features upon which to base a classification and, until more Green Bacteria have been investigated from this point of view, no definite statements as to the taxonomic position of most species can be made. In this connection it is significant that the yellowish color, regarded as characteristic of the Cyanochloridinae by Geitler and Pascher, is also met with in several true Myxophyceae. In the latter, contrary to Geitler's (1925, pp. 346, 362) opinion, it does not always constitute a specific feature but represents a state of nutrition (cf. p. 62), although some species may perhaps be more readily influenced in their pigmentation by nutritional deficiency than others. There is no doubt, as my cultures have shown, that minute, bacteria-like forms behave in this respect like larger Myxophyceae.

Until a better knowledge of Green Bacteria has been obtained, they may tentatively be placed in three groups: (1) Small unicellular Myxophyceae, 0.5 to 1 μ wide, usually referred to the Chroococcaceus genera *Synechococcus*, *Rhabdoderma*, *Dactylococcopsis*, *Cyanarcus*, *Chroostipes*, etc.; (2) Cyanochloridinae (Chlorobacteriaceae in Lauterborn's sense), such as *Tetrachloris*, *Pelogloea*, *Streptococcus varians* Ewart; (3) Green Sulphur Bacteria (in van Niel's sense), showing relation to the Purple Bacteria, e.g., *Chlorobium* Szafer, *Chloropseudomonas* Czurda and Maresch.

(1) The first group certainly does not belong to the Bacteria, since gliding movements are known in some of them (cf. p. 60), and the pigmentation seems to be the same as that of other Myxophyceae, with capacity of reversible bleaching under adverse conditions. Individual cells may not betray their color, owing to their small size, and may then easily be mistaken for true bacteria. It is only when these small forms are massed in large numbers that they can be identified by their blue-green hue fading to yellow. Some of them aggregate to form distinctive colonies.

(2) The Cyanochloridinae may still be nothing else than small Myxophyceae. Geitler and Pascher (l.c., p. 451) refer to a central colorless and a peripheral pigmented part of the cells and state that the yellowish forms are connected through intermediate shades with distinctly blue-green ones. These are features characteristic of Myxophyceae. The color of dense populations of organisms identified as *Pelogloea* and *Tetrachloris* is definitely blue-green.

(3) The Chlorobacteriaceae (Lauterborn in part) or Green Sulphur Bacteria (van Niel) share the habitat and certain characteristics with the Purple Bacteria. Attention was first drawn to them by Szafer (1911) and Nadson (1912). Skene (1914), who found them only in fresh water, grew them in cultures. Bavendamm (1924) found it difficult to exclude green bacteria from his enrichment cultures of Purple Bacteria. Van Niel (1931, p. 27) isolated Green Sulphur Bacteria also from sea water; they appear as a green sediment with a greyish tinge. According to him (1944, p. 61), they contain a chlorophyll-like pigment which is 'most certainly not identical with either the bacteriochlorophyll of Purple Bacteria or chlorophylls a and b', a statement apparently only based on Metzner's investigation (cf. p. 79).

The only species of Chlorobacteriaceae so far thoroughly investigated is the *Chlorobium limicola* of van Niel (1931, p. 66), who inclines to the belief that all Green Bacteria belong to it (1931, p. 72), but this is unlikely to be correct. Even if little weight is given to earlier statements, there are distinct differences between the form studied by van Niel and *Chloropseudomonas* Czurda and Maresch (1937, p. 123). The former is non-motile, and strongly polymorphic (l.c., p. 65), while even single individuals are green; the latter is always rod-shaped, motile with the help of polar flagella, and colorless individually though olive-green (not greyish) when massed. They seem also to differ physiologically.

The variability of van Niel's *Chlorobium* is remarkable. It exhibits coccoid forms, long rod- to club-shaped cells, loosely or closely wound spiral structures and large ellipsoidal or spherical shapes. This polymorphism surpasses that of

every other microörganism and should be confirmed by thorough scrutiny. It is not stated whether all the various forms represented in van Niel's fig. 8 (1931, p. 65), were simultaneously found or, if not, under what conditions they were produced.

While groups (1) and (2) may have to be merged, especially as Lauterborn's *Pelodictyon* could scarcely form nets without gliding movement, which would show it to be a member of the Myxophyceae, group (3) is definitely not related to either of the others.

b. Purple and Brown Bacteria. The differences in color between purple, red, and brown bacteria are insignificant, changes being observed even in the same strain (van Niel, 1944, p. 4), so that all these organisms can be treated under the designation of Purple Bacteria. The storage of sulphur in the cells is also not quite as distinctive a feature as was previously believed (van Niel, 1931).

At first sight Purple Bacteria seem to afford little evidence of affinity to Myxophyceae, but a few aspects must be discussed. The possession of photosynthetic pigments, not contained in specially differentiated protoplasmic bodies (plastids), is common to both classes. The appearance of the cytoplasm of the large Purple Bacteria (e.g., *Chromatium okenii*) recalls that of certain Myxophyceae (e.g., *Chroococcus turgidus*) in its fine punctuation and apparent homogeneity and differs from that of other small organisms. Fischer (1897a) failed to demonstrate a central body like that of Myxophyceae in *Chromatium*, while Dangeard (1909) claims to have seen it, although he states that the pigments are diffused throughout the cytoplasm. This also seems to be so in *Chroococcus*. (Acton, 1914, p. 451; Geitler, 1936, p. 6).

These similarities would not amount to much, if all Purple Bacteria possessed flagella. Van Niel (1944, pp. 17, 81) shows convincingly that the polar flagellation of the motile forms, combined with the absence of spores and the gram negative character, admits of a reference of these forms to the Pseudomonadaceae (Kluyver and van Niel 1936). This involves abandoning the family Rhodobacteriaceae and separating certain non-flagellate Thiorhodaceae (Purple Sulphur Bacteria) from the others, although this conclusion was not drawn by van Niel. This author has recently raised the group, of which *Pseudomonas* is the type, to the rank of an order, the Pseudomonadales (van Niel, 1944, p. 82) with which one may well agree. This order is divided into the two families Pseudomonadaceae and Spirillaceae. The inclusion of the sulphur-containing forms in the Pseudomonadales is tacitly assumed by van Niel (l.c., p. 81), although no system of classification is given for the order.

The flagellate Purple Bacteria would apparently belong in part to the Pseudomonadaceae, in part to the Spirillaceae, each including sulfur-depositing species, as well as others which do not deposit sulfur. The Thiorhodaceae, however, comprise genera which cannot be included in the Pseudomonadales. Lauterborn (1915, p. 427) gives a table showing the morphological parallelism of certain colorless, red, yellowish green and blue-green microörganisms. The similarity between the colorless *Lampropedia*, the red *Thiopedia*, and the blue-green *Merismopedia* is striking, and so is that between the red genera *Thiodictyon* and *Pelo-*

chromatium on the one hand, and the yellowish green *Pelodictyon* and *Chlorochromatium* on the other. As in *Pelodictyon* (cf. p. 81), the arrangement of the cells in *Thiodictyon elegans* (Winogradsky, 1888, p. 80; cf. also Bavendamm, 1924, p. 124; Ellis, 1932, p. 169) is scarcely possible without the assumption that the cells can glide over one another. These organisms form net-like colonies of rod-shaped cells, originating by a gradual orientation from a dense primary aggregate. Under unfavorable circumstances the nets again contract into compact masses of cells. Reproduction of the colonies is effected by division or by breaking up into small, slowly moving cell aggregates.

It is not known whether the tabular colonies of *Thiopedia rosea* (Winogradsky, 1888, p. 85; photograph in Pringsheim, 1932a, p. 481) move like those of *Merismopedia* (cf. p. 60), but Winogradsky states that the cells of the former can scatter—perhaps in the same way as those of *Holopedia*? Another genus of Thiorhodaceae, *Amoebobactor*, of which three species have been recorded (Winogradsky, 1888, p. 71; cf. also Skene, 1914; Lauterborn, 1915, p. 424; Bavendamm, 1924, p. 122; Ellis, 1932, p. 168) seems likewise to exhibit gliding movements. According to Winogradsky the cells of the colonies are joined by invisible protoplasmic strands, although Lauterborn (1915) could find no indication of them. The cells are described as globular or ovoid and as capable of altering their shape (Ellis, 1932, p. 168). The colonies slowly change their appearance as the cells become closely approximated or separate from one another. Movement due to elastic threads seems to be merely a hypothesis of Winogradsky's, but there is also no real evidence of ameboid movement. It is more likely to be of the same mysterious nature as that of other gliding organisms.

If the occurrence of gliding movements in *Thiopedia*, *Thiodictyon* and *Amoebobacter* be confirmed by further observations, there would be two independent groups of Purple Bacteria, viz., I. Rhodopseudomonadaceae with the subfamilies Athiorhodeae and Thiorhodeae, differing in the deposition of sulfur outside or inside the cells, and in the ability of the latter to live autotrophically; II. Amoebobacteriaceae, sharing with the Thiorhodaceae the storage of sulfur, but awaiting investigation as regards their pigmentation and mode of nutrition. The latter family would perhaps be related to the Cyanochloridinae.

The Purple Bacteria would thus appear as a mere physiological group comprising unrelated forms, although they are not spread over the whole of the Bacteria, as Molisch (1907, p. 26) suggested would be the case if they were not defined as a natural group characterized by its pigmentation.

4. Spirochaetae. Cohn (1853, p. 132) says: 'Just as the colorless *Spirochaeta plicatilis* was found to correspond to the blue-green *Spirulina*, although living only in putrid infusions, so, by investigating fermenting fluids, other forms are encountered which, though unpigmented, have their nearest relations among the algae.' The parallelism between *Spirulina* and *Spirochaeta* has often since been discussed, without finding many advocates. The differences, apart from that of pigmentation, customarily stressed are: (1) *Spirochaeta* is unicellular, *Spirulina* multicellular; (2) *Spirochaeta* has an axial cord, *Spirulina* lacks it; (3) *Spirochaeta* is flexible, *Spirulina* rigid; (4) some *Spirochaetae* swim freely, while *Spiru-*

lina, when moving, always adheres to some solid surface. Most of these matters cannot be fully discussed, because the investigation of these small organisms, and particularly of the delicate spirochaetes, has not so far yielded sufficient evidence (Topley and Wilson, 1946).

The multicellular structure of some of the smaller species of *Spirulina* is not conclusively established. The neutral red stain used for this purpose does not give clear results. A filament may display a row of chambers without being multicellular in the strict sense. The distinction between *Spirulina* without, and *Arthrospira* with cross-walls is in doubt. The grouping of the smaller species as (*Eu-*)*Spirulina*, and the wider ones as *Arthrospira* suggests that the recognition of cross-walls depends on the width of the trichomes (Schmid, 1921; Crow, 1927). Moreover a cellular organization has not the same significance in Myxophyceae as it has in other algae (cf. pp. 53, 66).

Dyar (1947, p. 491; fig. 17, p. 489) has demonstrated axial cords in *Spirulina* and gives reasons for regarding them as artefacts. They are moreover not always found in *Spirochaeta*, not even in electron micrographs (Morton and Anderson, 1942; van Thiel and van Iterson, 1947), so that some at least of the older claims seem to be founded on misinterpretation.

Flexibility is an important characteristic which must be discussed in some detail. In its relation to gliding movement, it is not only an essential distinction between *Spirochaeta* and *Spirulina* (Zuelzer, 1910; 1911, p. 45), but also has some bearing on the general question of possible relationship between Bacteria and Myxophyceae. Although frequently mentioned in this connection it does not seem that flexibility has ever been properly defined. If it meant merely liability to change of shape as a result of external forces, it would be identical with pliability which is inherent, though in various degrees, in any physical body. *Spirillum*, for instance, is supposed to be rigid, but long spirals are easily deformed by traction (Hama, 1933, pp. 137/38). Zacharias (1903 b) describes a large *Spirillum* (*Pseudospirillum uliginosum*) as flexible, while Swellengrebel (1909, p. 548) and Meirowsky (1914, p. 61) sometimes observed in *Spirillum* flexibility like that of *Spirochaeta*. Such spiral organisms, apart from their pliability, show also a certain degree of elasticity. 'The spirals of *Spirulina* can be drawn out until the thread is almost straight, but take on their previous form, when the tension is released' (Fritsch, 1945, p. 788).

If therefore the description of an organism as flexible is to have any significance, it can only mean that the body is especially liable to alter its form, either as a result of outer mechanical influences, or owing to the operation of internal forces. The second alternative which is apparently that assumed by most writers, not only on spirochaetes, but also on motile Myxophyceae, should be termed active flexibility.

Oscillatoria and *Beggiatoa*, for instance, give the impression that their trichomes are markedly flexible. When a mass of trichomes is transferred to a drop of water under the microscope, the loops and tangles caused by the surface tension of the water commence to straighten out, with the tips of the trichomes radiating in all directions, their curved ends exhibiting the well-known rotation. Threads

still in close proximity creep along one another and between particles of débris, apparently evading obstacles and following an outward path by active flexible movement. On closer inspection, however, it is clear that the straightening out of curvatures and loops is caused by elasticity, while bending to evade obstacles is due to passive pliability, as already stated by Correns (1897) and Kolkwitz (1897).

It seems to have been overlooked that the flexibility of *Spirochaeta* may perhaps be brought about by the same causes. The body is much narrower and much quicker in its movements. It turns in various directions at frequent intervals; the movement is characteristic for its restlessness and accompanied by strong bending or often even coiling; but that does not necessarily mean that its flexibility is really 'active'. Changes in direction may be caused by differences in external friction or by minute obstacles, and not by unequal internal forces operating on different sides. The unpredictable 'lashings about', which have impressed many observers, may be the result of the impact of imperceptible external impediments on an organism whose shape is readily transformed, and which partly adheres to the glass or other substrata. The difference in behavior, e.g., between *Spirulina* and *Spirochaeta* would then merely be such of degree and not of the nature of the movement, and mainly due to differences in width and rigidity. As an illustration one may compare the mechanical properties of a narrow, elastic, closely coiled wire with those of a wider, less closely coiled one. The former would easily bend in any direction, even through the impact of a stream of air, which would apparently not affect the latter.

While such a conception would not account for the gliding itself, it would help to explain the differences in the mode of motility of the two organisms. The question of course arises whether there is never any doubt as to the reference of a certain specimen to one or other genus. The decision is mostly easy, although *Spirulina* and *Spirochaeta* have so much in common that a general and fundamental difference is difficult to define, and this similarity is demonstrated by the existence of forms of doubtful affinity.

Lagerheim (1892) described two species of *Glaucospira* which, he thought, rendered the relation between *Spirulina* (Gomont) and *Spirochaeta* (Ehrbg) a certainty. These forms possess the characteristics of *Spirochaeta*, but are blue-green. They have narrow coils and form secondary spirals, so characteristic of spirochaetes. The organisms show lively to and fro movements, while, without apparent reason, the direction of movement suddenly becomes irregular, with almost spasmodic bending to different sides 'like a wounded snake'. That is just what is observed in *Spirochaeta*.

Dobell (1912) suspects that the bluish tint was the result of poor optical equipment, but this is not likely to be so. Such delicate organisms, the coils being only 2 μ wide, could scarcely show color individually, so that Lagerheim must have judged it by that of massed cells. However that may be, Skuja (1939, p. 48) describes an organism which he regards as identical with that of Lagerheim, although it is colorless. It is stated to exhibit the kind of movement characteristic of *Spirochaeta* so that it is not altogether clear how it is distin-

guished from that genus. The description and figure (pl. I, fig. 5) show a form very similar to *Spirulina tenuissima*. No final judgement is possible until Lagerheim's and Skuja's forms are found again. A colorless *Spirulina*, with narrow coils, has already been found by van Tieghem (1880) who describes it as hyaline without being a *Spirochaeta*.

5. Myxobacteria. The Myxobacteria resemble many Eubacteria in shape and size, while their movements show some similarity to those of Myxophyceae. Those Myxobacteria that were first discovered differ from both groups in producing peculiar fruiting bodies. Stanier and van Niel (1941, p. 464) in their discussion of bacterial classification, refer the Myxobacteria, together with the Eubacteria and the Spirochaetae, to the Schizomycetae which removes them rather far from the Myxophyta (Myxophyceae). Jahn (1924, p. 104) regards the Myxobacteria as apochlorotic Myxophyceae, a suggestion which does not seem to have been taken seriously by most authorities, although there is much that speaks in favor of this view.

The facts enumerated by Jahn in support of a relationship between Myxobacteria and Myxophyceae are few. He mentions as common to both the softness of the cell wall, the lack of a defined nucleus, the storage of glycogen, and the kind of movement which he regards as most important. *Oscillatoria* is considered unsuitable for comparison, because here the motion of large multicellular trichomes leads to a special organization which is also indicated by their rotation. This is lacking in the Myxobacteria, but also in the hormogonia of *Nostoc*, *Anabaena* and *Cylindrospermum* (Harder, 1918). For Jahn the decisive fact is the existence of forms intermediate between Myxobacteria and Myxophyceae, namely small unicellular blue-green species he found on moist soil and whose movements he describes as very similar to those of Myxobacteria. According to him such Myxophyceae repeat the kind of movement, the mucilage-exudation and the colony-formation of Myxobacteria. He was unfamiliar with the non-fruiting forms which show still greater similarity with certain Chroococcaceae. Geitler (1925, p. 172) rejects Jahn's view because no swarm-formation is found in the relevant Myxophyceae, although closer observation may still reveal it, while Stanier and van Niel (1941, p. 452) regard Jahn's claim to have observed creeping movements in unicellular blue-green algae, if substantiated, as providing strong support for his hypothesis. Such movements have in fact been repeatedly recorded, also by the present writer (cf. p. 60) so that there is no doubt as to the correctness of Jahn's statement. Microcyst formation, which is probably found in all Myxobacteria, but is lacking in Myxophyceae, is, however, another reason against a close relation between the two groups.

Soriano (1945) restricts the order Myxobacteriales to those Myxobacteria which possess fruiting bodies, while the non-fruiting forms, together with the Beggiatoaceae, are classed in the new order Flexibacteriales, which comprises the two families Flexibacteriaceae and Cytophagaceae. This classification is unnatural in two respects. The structure of the vegetative cells and the formation of microcysts are common both to the fruiting and non-fruiting Myxobacteria, while in the Beggiatoaceae the structure and arrangement of the cells is

different and microcysts are lacking. Soriano's separation of the cellulose-attacking Myxobacteria as the family Cytophagaceae from the remainder, the Flexibacteriaceae, lays far too much stress on a physiological feature of doubtful significance. Where, for instance, should one then class Stanier's (1947a) chitin-destroying *Cytophagas?* Stanier (p. 313) rightly says: 'if these organisms really comprise as large and varied a group as appears to be the case . . . careful thought should be given to the selection of significant, mutually exclusive differential characters.' He also emphasizes the difficulty arising from the complexity of biochemical properties in an attempt to use them for taxonomic purposes. A Cytophaga may be able to decompose cellulose but also to live on glucose.

A closer affinity of the Myxobacteria may exist to the Vitreoscillaceae than to unicellular Myxophyceae (cf. p. 70). Some species of *Vitreoscilla*, in the readiness with which they dissociate into short filamentous fragments and single gliding cells, as well as in their low refraction, seem to be more like Myxobacteria than like *Beggiatoa*. They might even, in spite of the absence of microcysts, be regarded as filamentous counterparts of the Myxobacteriaceae, as the Oscillatoriaceae are the filamentous counterparts of the Chroococcaceae, provided that additional evidence in support of this view were furnished by further investigation.

In this connection it is of interest that there are organisms resembling Myxobacteria but capable, like many Eubacteria, of forming multicellular trichomes; they have been isolated in pure culture. Garnjobst (1945) obtained such a Myxobacterium, which she called *Cytophaga columnaris*, from diseased fish; this grows well on peptone agar and in liquid media, is strictly aerobic and produces a carotene pigment which turns blue with sulphuric acid. It utilizes neither glucose nor lactose. The filaments may attain a length of more than 150 μ, forming almost mycelium-like colonies on agar (p. 124, fig. 5); microcysts were not found. Stanier (1947a, p. 314) proposes the name of *Flexibacter* for myxobacterial forms capable of growing in the absence of carbohydrates, and *Cytophaga* for those requiring them. Garnjobst's species should then be called *Flexibacter columnaris*. Stanier is probably right in not attaching much importance to the presence or absence of microcysts.

I have repeatedly isolated a similar organism on agar plates with 0.05% yeast extract from decaying plant residues and from cow dung. It exhibits two growth forms, sometimes spreading like a mycelium from the place of inoculation, at other times being denser with fringed edges and closely resembling *Cytophaga* as shown by Stanier's photographs (1947a, p. 310). The spread over the agar is readily influenced by small differences in moisture. The filamentous strands on agar are composed of thin rods which separate in mounting a preparation, while the appearance of turbidity in liquid media is due to the presence of irregularly bent filaments reaching 50 μ in length and exhibiting the slow, interrupted, hesitating movements characteristic of Myxobacteria, as well as of unicellular pigmented and colorless Myxophyceae and of Vitreoscillaceae.

In its nutritive requirements and the production of an orange pigment this common organism resembles *Flexibacter columnaris*, but it differs from it in its ap-

parent non-pathogenic character and in producing microcysts. Since the latter were only found in a few old liquid cultures, they may have been overlooked by Garnjobst, although microcysts are stated to be lacking in a number of other Myxobacteria (Garnjobst, 1945, p. 126) and were never found in the Vitreoscillaceae.

These filamentous Myxobacteria share with the Vitreoscillaceae the gliding movement, the low refraction, the appearance of the growths on agar, the kind of nutrition, and in part the production of carotenes. Whether they are really nearly related can only be established by further investigation.

V. DISCUSSION

From the consideration of facts given in this review, it emerges that there is no affinity between Bacteria and Myxophyceae. A similar opinion was expressed long ago by Chodat (1909, p. 135): '... il devient de moins en moins probable que ces deux classes (Schizomycètes et Schizophytes) soient réellement parentes.... Les deux groupes se ressemblent probablement bien plus en vertu de phénomènes de convergence, que grâce à une réele affinité naturelle.' While this view was based on the differences in cell structure (cf. p. 54), Geitler (1934, p. 185) seems to be impressed mainly by morphological differences when he says: 'Da die positiven Übereinstimmungen im feineren Zellbau andererseits nicht sehr gross sind, lässt sich auch die Auffassung der Blaualgen als eigener Pflanzenstamm (Cyanophyta) vertreten.' He is of the opinion, however, that the Trichobacteria may possibly have a closer affinity to Myxophyceae than other Bacteria, a point of view discussed above (p. 77). It does not lead us any further to admit that the two classes may perhaps have an extremely remote common ancestry because even of this there is no real evidence.

The denial of any relation between Bacteria and Myxophyceae applies to the bulk of the former, which are called Eubacteria in treatises dealing with the classification of the class. It may be doubted, however, whether the Eubacteria constitute a natural group, and their subdivision into three or more independent classes will probably become necessary. None of these would serve as a bridge to the Myxophyceae, although the Myxobacteria have the gliding movement in common with them; but they have nothing to do with true bacteria.

The reasons for and against an affinity between Myxobacteria and Myxophyceae have been discussed in detail (p. 85). The evidence speaks against a close relationship, but a common origin is quite possible. It is indicated by the kind of movement which suggests a similar cell organization. Moreover Myxobacteria and Myxophyceae are connected by way of the Beggiatoaceae and the Vitreoscillaceae. Certain of the latter show resemblances to Myxobacteria, while others are more like Beggiatoaceae and may therefore prove to be apochlorotic Myxophyceae. The Vitreoscillaceae seem, however, to be a natural group differing from the majority of the Myxobacteria in their pronounced filamentous habit, and in the absence of the microcysts characteristic of the latter.

These considerations bring one to the borderline of mere speculation, but one may be tempted to sketch, though only in dim outlines, two very large groups,

that of the gliding and that of the swimming organisms, neither of which can be postulated as more ancient than the other. The former include the Myxophyceae, the Myxobacteria, the Vitreoscillaceae, the Spirochaetae, while Rhodophyceae, Desmids and Diatoms, which also exhibit gliding movements brought about in different ways, do not belong here in view of their advanced cell-organization. The only classes containing members exhibiting both swimming and gliding movements, are the Chrysophyceae and the Euglenineae, and it is obvious that they have nothing in common with the Myxophyceae. Gliding movement in general presupposes a special cellular organization, namely soft membranes differing from the cell walls of Algae and Bacteria, and filaments with interconnection of cells by way of the septa, which are not true cross-walls and permit coordination between the cells, but resulting in death when part of a trichome is injured.

Swimming organisms possess flagella (or cilia) and the presence of these organs of locomotion cannot in general be regarded as a taxonomic character, since they are found in groups with no obvious affinity to one another: Bacteria, Spirochaetae(?), Chrysophyceae, Xanthophyceae, Phaeophyceae, Diatoms, Chloromonadinae, Euglenineae, Chlorophyceae, Charophyta, Bryophyta, Pteridophyta, Gymnospermae, Myxomycetes, Phycomycetes, and Flagellata of doubtful affinity, as well as Ciliata and most other animals including Mammalia. Unless one assumes a polyphyletic origin for flagellar locomotion, all the Flagellata and their descendants must be derived from remote common flagellate ancestors, from which the Bacteria, most Algae and Fungi, and other plants, as well as animals have descended, or alternately the Bacteria might stand near the origin of the majority of living organisms. There is, however, no clear connection between flagellate Bacteria (Holoplastideae) and other flagellate organisms (Euplastideae), such as Flagellata, Ciliata, etc., while the evolution of various classes of Algae from flagellates is widely accepted.

For the time being there is insufficient basic knowledge, even of relatively restricted groups like the Myxobacteria, Vitreoscillaceae and Myxophyceae on the one hand, and of the true Bacteria on the other, to justify the utilization of the mode of locomotion on a broad scale as a diagnostic character for taxonomic purposes, for which it might well become useful in future. The scanty evidence available for assessing relationship could probably be supplemented by cytological details brought to light by improved staining methods, and with the help of the electron microscope, as well as by better biochemical characterization of the nature of the relevant cell constituents, cytoplasmic, nucleoproteid, etc., including serological tests. The latter have been somewhat discredited by their uncritical application in certain quarters to problems of taxonomy. Far-reaching conclusions cannot be expected to be trustworthy, if the same technique is applied for large groups as has proved to be valuable in the comparison of closely related forms. Modern methods of extracting specific proteins and other compounds of high molecular weight may eventually afford the clue to the problems above indicated.

While a near affinity between true Bacteria and Myxophyceae cannot be ac-

cepted, there still remains the problem of the relation between pigmented and non-pigmented forms of comparable organization. This involves the question which bacteria-like organisms should be considered as apochlorotic Myxophyceae and which as Bacteria. It is possible too that present-day Bacteria include apochlorotic Myxophyceae which still await detection although this is so far only a hypothesis.

When two sets of organisms differ in the presence or absence of photosynthetic pigments, but are otherwise similar in all essential respects, the phenomenon is usually explained as due to a phylogenetic connection between the two. Pigment can either have been lost or have been acquired, after the principal characteristics of the organisms had become fixed. The pigments of the chlorophyll-group possess complex chemical structures and, together with other pigments, form a photosynthetic system which is mostly located in special parts of the cytoplasm. The whole mechanism is too complicated to have developed more than once in the same way. The Purple Bacteria and the Green Sulphur Bacteria (cf. p. 80) differ in this respect from the majority of photosynthetic organisms, and this indicates that they belong to an independent line of evolution.

By contrast loss of the photosynthetic apparatus can easily be supposed to have occurred in various instances, provided that the organisms concerned were able to survive the change in nutrition, owing to their possession of other means of acquiring food.

In considering the general relation between Bacteria and Myxophyceae the question whether colorless or pigmented forms of similar construction are primary can only be put if the organisms in question are clearly related. It can therefore only be propounded with regard to those 'Bacteria' which can be definitely recognized as apochlorotic Myxophyceae, i.e., *Beggiatoa*, *Thioploca*, *Thiothrix*, *Achromatium*, *Vitreoscilla*, etc.

Benecke (1912, p. 243), when discussing the relation between certain Bacteria and Myxophyceae, leaves the question of origin open: 'Which are the more primitive, colorless bacteria or pigmented algae, is a question about which one can dispute to one's heart's content, as nothing certain is known.' Stanier and van Niel (1941, p. 450) say: 'Thus is seems at least possible that the primitive blue-green algae of the *Chroococcus* type have developed from the Eubacteriales as a second photosynthetic line' (apart from the Purple Bacteria). Even if the possibility of such an affinity were accepted there is in the data they give little evidence of the direction of the evolution.

Van Niel (1944, pp. 80 *et seq.*), in attempting to determine the taxonomic position of Purple Bacteria, cites the relation between Flagellata and Algae and their colorless counterparts, as a parallel to that between pigmented and unpigmented Bacteria. The Purple Bacteria are, he states, 'polarly flagellate' (cf., however, p. 81), 'they fall . . . in the morphological family of the Pseudomonadaceae, and represent the pseudomonas, vibrio, and spirillum types, resembling completely the non-photosynthetic members of these tribes.' He includes therefore all pigmented bacteria in the order Pseudomonadales. Since the transformation 'from pigmented to non-pigmented forms' (of algae) 'but

not the opposite, has been experimentally achieved, it appears more logical to consider the purple bacteria as the progenitors of the corresponding non-photosynthetic bacteria' (so already Delaporte, 1940).

There are some more conclusions which were not drawn by van Niel. If photosynthetic bacteria are more primitive than colorless ones, they must have acquired their pigments before morphological diversity had developed. The main evolution of the pigment-free Pseudomonadales, up to the Chlamydobacteriaceae, would have taken place after the loss of pigments, and they would not be related in any way to the rest of the true Bacteria which must have had a different origin. The gap would be so wide as to split the 'Eubacteria' into two groups which would better be considered as independent classes of the same status as the Myxobacteria for instance and might be called Bacillales and Pseudomonadales. The resemblance between the two would be the result of convergent evolution. The Bacillales are less homogeneous than the Pseudomonadales; the position of the Coccaceae remains undecided owing to doubts on their homogeneity, while the Myxobacteriales are again an independent class.

Van Niel's conclusion as to the origin of colorless bacteria is based on a comparison with what is known to occur in other groups. The direction of evolution in these is indicated by the scattered occurrence of unpigmented forms among a majority possessing a functioning photosynthetic apparatus (Pringsheim, 1941, p. 193). Apochlorosis has clearly originated here and there in a small number of taxonomic groups which, by virtue of their special modes of nutrition, were able to survive the loss of assimilatory pigments.

The same conclusion can be drawn for colorless Myxophyceae. That is the answer to Benecke's question, while the one propounded by Stanier and van Niel remains open until unicellular colorless forms are found, which in conformity with their concept, are bacteria-like, non-motile, coccoid and gram negative, and which may be suspected with some justification to be derived from Chroococcaceae, although they are not recognized with certainty as such any more. Whether, e.g., a four-celled 'colorless *Chroococcus*' mentioned without details by Bavendamm (1924, p. 45) belongs here, cannot be ascertained. Members of the Achromatiaceae would not be of this nature, since they are recognizable as apochlorotic Myxophyceae by their kind of movement. *Gonococcus* and *Meningococcus* are probably too far modified by parasitism to come into question. The great majority of Coccaceae can be ruled out because they are gram positive. There is therefore no indication that any member of the true Bacteria has originated from Myxophyceae.

VI. SUMMARY

I. The theory of an affinity between Bacteria and Myxophyceae has recently been revived by regarding the former as an apochlorotic offshot of the latter, an interesting but controversial proposition.

II. In order to clarify the position, certain aspects of bacterial taxonomy are revised. By using all available means of recognizing natural relationships the Bacteria can be divided into a number of independent classes, none of which could have evolved from Myxophyceae.

III. This conclusion is founded on a review of the various characteristics of the Myxophyceae and a comparison of them with those of the Bacteria. Morphological similarities appear more probably to have originated by convergence and afford no evidence of descent from a common ancestry. The pigmentation, where present, is different; the cytological and physiological characteristics are not altogether comparable; motile Bacteria swim freely, while Myxophyceae glide along solid surfaces. Some so-called Bacteria, which do not display the usual characteristics of true Bacteria, can be shown actually to be pigment-free Myxophyceae.

IV. Apochlorotic Myxophyceae, which are reviewed in some detail, are more numerous than is generally believed. Among accepted Myxophyceae there are very few apochlorotic species and these in part are doubtful, but *Beggiatoa* in all its characteristics shows a close relation to *Oscillatoria*, while *Thiothrix* and *Achromatium* can also be recognized as colorless Myxophyceae. A number of other pigment-free forms have been discovered (and some of them grown in pure culture) which, by their gliding movements and other features, can justifiably be grouped among apochlorotic Myxophyceae.

V. Other bacteria-like organisms which, for one reason or another, have been suspected of being related to Myxophyceae, are discussed in this connection. Certain green and purple bacteria, spirochaetes, and myxobacteria have at least the characteristic of gliding movement in common with Myxophyceae, while the remainder belong to the Bacteria proper.

VI. The type of movement as a taxonomic character is discussed and supplemented by other features so that some progress towards a more natural classification is reached. Problems connected with the direction of evolution from pigmented to colorless organisms are discussed.

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INFLUENCE OF NUTRITION IN EXPERIMENTAL INFECTION[1]

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"What is one man's meat is another man's poison" (1) is demonstrated daily in every medical clinic. Although clinical observations as well as general impressions suggest that optimum nutrition commonly aids resistance, our knowledge of nutrition is still too fragmentary to give an adequate statement of "optimum nutrition" for persons living under markedly diverse conditions "from Greenland's icy mountains to India's coral strand," and our knowledge of the factors involved in resistance seems even less complete. Even though we can suggest diets for various living conditions with a fair degree of success, the world is far from providing even an approximation of these diets to millions of the members of our family. Malthus is riding hard and fast with all four of the horsemen of the Apocalypse.

It is recognized that what appears to be abundant health under apparently admirable living and dietary conditions, does not protect against the common diseases of childhood, measles, chickenpox, or mumps, nor against many diseases of adult life such as the common cold and influenza. Ill health and poor nutrition do, however, appear to increase the incidence of such diseases as rheumatic fever and tuberculosis.

[1] This review stems from a "Round Table" held at the annual meetings of the Society of American Bacteriologists at Minneapolis, May 1948.

Cyrus P. Barnum, Jr. and David Glick, both of the Department of Physiological Chemistry of the University of Minnesota Medical School took part in the discussion but did not present papers and are not therefore involved in this review.

Paul F. Clark was the convenor of the "Round Table" and has served as editor of these papers.

What then is the evidence pertaining to the influence of nutrition on susceptibility or resistance in the more adequately controllable experimental infections? We shall interpret the term nutrition quite broadly, to include not only the utilization of commonly recognized foodstuffs, but also any chemical compounds which are known to affect cellular metabolism. Our consideration will necessarily be incomplete and devoted chiefly to the results of recent studies. With surprise at our own temerity in tackling so complex a problem, may we suggest certain "ground rules" which we would lay down for ourselves and which we should hope might be followed by all persons working in this field.

1. The fundamental importance of the genetic constitution both of the host and of the parasite have frequently been overlooked; we would stress the necessity of considering these genetic factors in most animal experimentation. We would suggest that universities combine in building up stocks of all the common experimental species of known genetic constitution. Each animal would cost more but the quality of the results would be markedly improved.
2. The choice of experimental model is always difficult and never free from objection. A disease spontaneous in the species under study is usually preferred; frequently, however, if the parasite from man can be transferred to another species with the production of a disease somewhat similar to that in man, this becomes a favored model, for example, Lansing strain poliomyelitis in mice.
3. The natural portal of entry can rarely be employed although if possible it is preferred over an artificial route. Frequently the route chosen must be highly artificial involving more or less trauma. The results from different routes of injection may be compared, but never directly.
4. In deficiency experiments, basal rations of known chemical composition should be used if possible, with varying levels of the particular component under study and with especial attention to an adequate intake of all essential factors except those deliberately restricted. With low levels of the factor, possible competition between host and parasite must be considered.
5. Not infrequently, the animals on experiment are suffering from complex deficiencies, dehydration, etc., at the time of the challenge infection. The distinction should be maintained between inanition, a quantitative depletion due to ingestion of an insufficient amount of an otherwise adequate diet, and malnutrition in which the caloric intake may be adequate but qualitatively a deficiency of one or more essential dietary components exists.
6. Age, weight, sex, segregation of litter mates, physical condition of the cages, likelihood of coprophagy, variation in intestinal flora, temperature and humidity of the laboratory should be and usually are well appreciated at the present time.
7. Consideration must be given to reports on dietary influence on protein synthesis, antibody formation, phagocytic activity, etc.
8. Manifestly, experimental groups of animals should be sufficiently large to provide statistically significant figures. A consistent tendency in several smaller series may, however, be even more convincing.

9. Uninfected animals on the deficient diet and a group injected with the uninoculated medium or normal tissue are controls too frequently omitted.
10. Since nutrition studies commonly extend over a long period, the virus or other parasites should as far as possible be frozen, or frozen and dried in quantity to serve as a basis for fresh material in the continuing experiments. Too many alterations in virulence or other characteristics may occur if serial passage virus or repeated transfer cultures are employed.

THE GENETIC BASIS OF NUTRITION EFFECTS IN NATURAL RESISTANCE TO INFECTION

Howard A. Schneider

In the understanding of how nutrition may influence an infection, we are, I believe, all agreed that nutrition is logically considered an environmental factor, and by introducing the necessity of dealing with genetic constitution, or the influence of heredity, those tired and venerable ghosts which plague every biological banquet, Heredity and Environment, immediately rise to their feet. It is our purpose, if possible, to show that these are not two antagonists, but only two aspects of a single phenomenon, the Natural World.

First, may I present evidence which leads us to begin a discussion of nutrition with facts about genetics. In the natural world infectious disease is a population problem. As our methods of study have improved we have become increasingly aware that the pathogens, like the poor, are with us always. It is also true, and a matter of direct observation, that when infectious disease makes it appearance, only certain members of the population are affected while others, in apparently the same circumstance, remain unaffected. Why is this so? The experimental epidemiological studies of Topley, Greenwood and Wilson in London and Webster in New York answer this question in part. Using herds of mice and natural diseases of mice these two groups sought by direct experiment to unravel the events in epidemic phenomena. The London School made many important contributions which it would be an impertinence to cite here in view of the detailed summary that group has published (2). Our present necessity, however, may drive us to say that, by and large, the London School concerned itself with the epidemiological consequences of the spatial and temporal arrangements of hosts and pathogens, and their studies demonstrated the important influence on epidemic disease of such factors as population densities, host immigration rates, the assembly and dispersal of herd units, etc. This spatial-temporal analysis, although it helped to formulate, and demonstrated experimentally, certain epidemiological concepts, did not, in my opinion, fully answer the fundamental question of the diversity of disease response in an exposed population. It was Webster who successfully faced the question of survivorship and made an analysis which applies here. Why, in these herds, did certain mice die and others live? Is it because of unequal dosage and hence different risks of exposure? No. Webster showed that identical doses, directly administered, produced the same diversity in disease effects (3). Is it because some mice first encountered, by chance, a "small immunizing dose" and thus were able to survive the subsequent, larger, average dose experience of the herd? No. In direct experiments with

mice and mouse typhoid Webster (4) was never able to demonstrate the reality of the "small immunizing dose" (For an opposite view see Greenwood *et al.*, 2.)

Webster turned to the hypothesis that the variation in the disease response was due to variation among the mice. This is a fundamental recognition that the description, "a herd of mice," is an inadequate one to be applied to the host in experiments in infectious diseases. But if the mice varied, what was the cause of that variation? Heredity? Environment?

Webster elected to examine the case for heredity. By inbreeding and selecting from a common stock of mice he was able to extract two strains of mice which were widely divergent in their response to infection with *Salmonella enteritidis*, one strain exhibiting almost complete survivorship, the other almost complete mortality (5). By performing this basic operation of the geneticist, inbreeding and selection, Webster was able to arrange the hereditary material and perpetuate stocks of mice which were, from generation to generation, predictably resistant or susceptible, i.e., resistance and susceptibility were *capable* of being brought under genetic control. Tested under herd conditions, by assembling herds of different percentile compositions of these two stocks, these relationships held true. Resistant stocks survived and susceptible stocks died (3).

We must bear in mind the operation, inbreeding and selection, which was performed to obtain these stocks. Those finally obtained serve to demonstrate, not that resistance and susceptibility are always or necessarily under genetic control, but rather that, if we do as the geneticist does, we may bring them under control which we label genetic.

In later work, Webster showed that resistance or susceptibility to mouse typhoid was independent of resistance or susceptibility to a second experimental infection, St. Louis encephalitis (6). This independence made possible the arranging of four different stocks of mice illustrating the four possible combinations of resistance or susceptibility to two different diseases. The status of these stocks is evident from tests performed in 1942 (7) and presented here in table 1. I believe that this demonstration decisively demolishes any notion we might have of resistance, as an attribute of "health," to all infectious diseases. In one and the same animal we may have resistance to one disease and susceptibility to a second disease.

We have emphasized that the demonstration of the genetic factor does not thereby exclude the operation, and even importance, of other factors. We must, however, grant genetics a position of primary importance and we maintain that this fact is frequently neglected in experimental studies. The long time operation of the selective effects of disease tends to drive the control of these events into the germ plasm, so to speak, and thus to escape the vicissitudes of the environment. If, by evolution, this process is complete, we recognize the *fait accompli* and do not waste our energies further. We are not, for example, considering the effect of nutrition on the occurrence of blue or brown eyes, or of blood groups. In the case of the infectious disease, the wide range of variation so often met in the disease response indicates, I believe, that the evolutionary process is still in

progress. Therefore, we need not throw up our hands and resign all to the geneticist. But neither should we ignore the fact that at least some of the evolutionary process has preceded us in time. No, if there is any hope that the slow grinding mill of evolution has not yet ground all the meal, we must recognize that as nutritionists, as environmentalists, we must deal with the situation as genetics has left it at this spot in evolutionary time. This is why genetics occupies so pivotal a position among the biological sciences.

Before we leave this brief description of the role of genetics in infectious disease, I should like to mention that whenever the influence of genetics has been analyzed in human populations with appropriate techniques, its role has been made evident. This is true for infectious disease of such diverse etiology as leprosy (8, 9), rheumatic fever (10, 11), poliomyelitis (12), and tuberculosis

TABLE 1‡

Survivorship of inbred strains of mice selected for resistance or susceptibility to infection with S. enteritidis or St. Louis encephalitis virus

DESCRIPTION OF STRAIN	STRAIN DESIGNATION	TEST PATHOGEN	NO. OF MICE TESTED (IN 1942)	SURVIVORSHIP*
				per cent
Bacteria-resistant, virus-resistant	BRVR	*S. enteritidis*	449	92
		St. Louis encephalitis	606	76
Bacteria-susceptible, virus-resistant	BSVR	*S. enteritidis*	86	2
		St. Louis encephalitis	98	88
Bacteria-resistant, virus-susceptible	BRVS	*S. enteritidis*	287	82
		St. Louis encephalitis	519	2
Bacteria-susceptible, virus-susceptible	BSVS	*S. enteritidis*	285	2
		St. Louis encephalitis	271	3

* Testing dose: *S. enteritidis*, 5,000,000 by stomach catheter; St. Louis encephalitis, 10^{-2} suspension of infected mouse brain, intranasally.

‡ Adapted from Schneider and Webster (7).

(13, 14). One of the best of these studies is that of Kallman and Reisner (14) in tuberculosis. Table 2, taken from these authors, shows in summary form the effect of blood relationship on tuberculosis morbidity as studied by the twin method. These data comprise one of the most telling arguments to bring to our full attention the genetic factor in infectious disease. For example, if one member of a twin pair over 14 years of age develops clinical tuberculosis, then the chances are 2–3 out of 10 that the other twin will develop tuberculosis if the second twin is a dizygotic co-twin, *i.e.*, non-identical. But, if the twin relationship is monozygotic, *i.e.*, identical, then the chances of the co-twin developing tuberculosis rises to 9 out of 10. These are sobering facts.

If we grant the fundamental importance of genetics, but are insistent that we ought at least try to examine the possible influence of nutrition, we can make a beginning by considering the various genetic possibilities, both for host and

TABLE 2

Tuberculosis morbidity rates in the twin index families

	GENERAL POPULATION* OVER FOURTEEN YEARS OF AGE	RELATION TO TUBERCULOUS INDEX CASES					
		Husbands and wives	Parents	Half-siblings	Full siblings	Dizygotic cotwins	Monozygotic cotwins
Number of cases							
Cases of tuberculosis	—	14	114	4	136	42	48
Rates of reference							
Uncorrected	—	226	688	42†	720†	230	78
Corrected	—	197.5	676	33.5	534	164	55
Morbidity rates							
Uncorrected for differences in age	1.08	6.2	16.6	9.5	18.9	18.3	61.5
Corrected (Weinberg method)	1.37	7.1	16.9	11.9	25.5	25.6	87.3

* Estimated for a population in which the ratio of white to nonwhite persons approximates 5:1.

† All individuals above the age of fourteen.

Courtesy of Kallman, F. J. and Reisner, D., 1943, *American Review of Tuberculosis*, 47, 549.

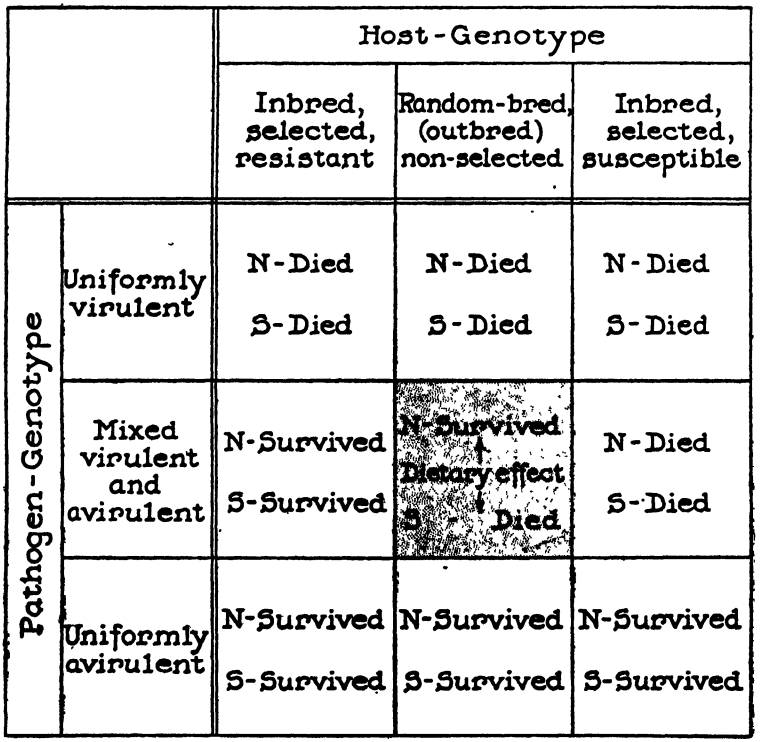

FIG. 1. The effect of a natural (N) and a synthetic (S) diet on survivorship following infection in nine different genetic circumstances.

From H. A. Schneider, Transactions of the New York Academy of Medicine, 1948 Eastern States Health Education Conference. (By permission.)

pathogen, under which nutrition might work. If we do this (7, 15, 16), we find that we can distinguish three genetically different kinds (genotypes) of hosts and three genetically different kinds (genotypes) of pathogen populations. Thus hosts are either (*a*) inbred (genetically uniform) and resistant, (*b*) inbred (genetically uniform) and susceptible, or (*c*) outbred (random-bred, genetically heterogeneous) and non-selected. Pathogen populations are also composed either of (*a*) uniformly virulent particles, (*b*) uniformly avirulent particles, or (*c*) a heterogeneous mixture of both kinds of infectious particles. In these statements we can see the attempt to encompass the facts of genetic variation. We can also see that host and pathogen can meet in 3 × 3 or nine different kinds of meetings. In which of these circumstances can we affect the mortal outcome of the infection by manipulating the nutrition of the host?

Before we attempt to answer that question, we are faced by the necessity of deciding what kind of nutritional manipulations we choose to make. The number we have to choose from is enormous, but let us follow the historical precedent. Let us inquire whether in the gap between present day knowledge of nutrition and the world of natural foodstuffs there may exist entities which our tests will reveal.

So if we compare (7, 15, 16) a diet of natural foodstuffs, whole wheat plus whole milk with a synthetic diet which contains all the desirable compounds we know about, and make this comparison in the nine genetic instances we have discussed before, we shall have at least one answer to our question. In figure 1, for mice and mouse typhoid, this answer is presented. I could go on to tell you about this survivorship principle revealed in this ninth instance, and failing to be revealed in the other eight, how it is present in the germ of wheat and go on to list its chemical properties as we have learned them thus far. For the present, however, we are content to make this point—that in the field of infectious disease, nutrition of the host operates in a genetic framework and the area of its operation is definable as the area in which genetically heterogeneous hosts meet with genetically heterogeneous pathogen populations.

THE INFLUENCE OF NUTRITION ON RESISTANCE TO EXPERIMENTAL INFECTIONS WITH HELMINTH AND WITH PROTOZOAN PARASITES

William Trager

The experiments of Ackert, McIlvaine and Crawford (17) with the intestinal nematode *Ascaridia lineata* of the chicken were among the first to indicate a relationship between a specific dietary factor in the nutrition of an animal and the degree of resistance of the animal to an infectious agent. When 7 week old chickens were placed on a diet deficient in vitamin A, they showed definite signs of deficiency 3 weeks later. If these chickens and non-deficient control birds receiving the same diet supplemented with cod-liver oil were then infected with counted numbers of eggs of *A. lineata*, the deficient chickens subsequently showed more and larger worms than the non-deficient ones. Weakened peristalsis and the larger amount of feces present in the intestines of the deficient chickens, thus perhaps providing a better food supply for the worms, were suggested as possible

explanations for the observed effects of the deficiency. Within the next few years, instances were reported in which vitamin A deficiency interfered with the development of acquired immunity in rats to the intestinal nematode *Nippostrongylus muris* (18), with the resistance of rats to both initial and secondary infection with *Trichinella spiralis* (19), with the resistance of dogs to two species of ascarids (20), and with both the natural and acquired resistance of rats to the nematode *Strongyloides ratti* (21). McCoy (19), observed that rats on a vitamin A deficient diet showed a lowered resistance to infection with *Trichinella spiralis* several weeks before the appearance of any other signs of avitaminosis. While in normal rats adult trichinae live only two weeks, in the vitamin A deficient rats they survived as long as their host—up to 57 days. Lawler (21) found that the resistance to strongyloides infection of rats on a vitamin A deficient diet was not decreased until the stores of vitamin A in the liver had been completely depleted. When this condition had been reached, resistance both to primary and to secondary infections with *S. ratti* was significantly lowered.

Deficiency in either thiamine or riboflavin lowers the resistance of rats to *Nippostrongylus muris*, another nematode parasite (22). The effect was slight in a primary infection but marked in a superinfection, suggesting that the deficiencies interfered primarily with antibody formation. Protein deficiency also interferes with the development of acquired immunity to this parasite (23).

Larsh (24) found that increased development of the cysticercoids, or larval forms, of the tapeworm hymenolepis occurred in mice which were partially fasted or were given alcohol. If the fasted or alcoholic mice were given a vitamin mixture, their resistance was as great as that of control normally fed mice, although their food intake was as low as that of fasted or alcoholic mice not given the vitamin supplement.

Much of the experimental work on nutrition and resistance to protozoan infections has been done with representatives of two genera of parasitic protozoa, the genus *Trypanosoma* which typically develops within the fluid portion of the blood of its host, and the genus *Plasmodium* which undergoes a major portion of its developmental cycle within the red blood cells of its vertebrate host. Manifestly, these two genera include some of the most important infectious agents of man and domestic animals, members of the genus *Plasmodium* being responsible for malaria, and the trypanosomes for African sleeping sickness and Chagas' disease of man and for nagana and related diseases of cattle and horses.

With the protozoa, as with other types of infectious agents, two opposite effects of nutrition on resistance are possible, and both have been observed. Let us consider first those instances in which a nutritional deficiency rather unexpectedly appears to enhance resistance to a protozoan parasite. Reiner and Paton (25) reported that rats on a diet deficient in the vitamin B complex survived infections with the blood flagellate *Trypanosoma equiperdum* slightly longer than did control animals on an adequate diet. Becker (26) observed that rats fed a heated diet and infected with the intestinal coccidian parasite *Eimeria nieschulzi* excreted in their feces fewer oöcysts than did rats which were fed the

same diet but not heated. The number of oöcysts excreted was increased, indicating a more severe infection, if the heated diet was supplemented with pantothenic acid. Similarly, if rats were fed a poor diet and were infected with eimeria, a supplement of calcium pantothenate did not improve the growth of the rats, but it did favor the growth of the parasites, as evidenced by increased oöcyst excretion (27). The addition of pyridoxine alone had a similar effect, but if thiamine was added together with the pyridoxine even fewer oöcysts were produced than in controls fed the unsupplemented diet (28). The feeding of thiamine together with pantothenate, however, had no such effect on the growth-promoting potency of pantothenate for eimeria.

Calcium pantothenate favors the survival of a bird malaria parasite (*Plasmodium lophurae*) in suspensions of duck or chicken red blood cells maintained *in vitro* (29). The results suggest that a deficiency of the host in patothenic acid may affect adversely the development within it of malarial parasites. This was found true in blood-induced infections of chickens with *Plasmodium gallinaceum* (30). In these experiments the chickens were inoculated when marked differences had appeared between the supplemented and the deficient groups. Whereas the infections in the chickens receiving pantothenate reached the usual peaks with over 50% of the erythrocytes parasitized, the infections in the chickens deficient in pantothenate showed only 3% or less of the erythrocytes infected.

Riboflavin, like pantothenic acid, appears to be even more essential to the life of certain avian malaria parasites than to the life of their host (31). When chicks fed a diet low in riboflavin were infected with *Plasmodium lophurae*, they subsequently developed infections with a peak parasite number only about one-fifth as high as that reached in control chicks on an adequate diet. Chicks which were fed the adequate diet but in restricted amount, so that they grew as poorly as the chicks on the riboflavin-deficient diet, developed even heavier infection than the fully fed controls. Although riboflavin deficiency made chicks relatively unsuitable for the growth of malaria parasites, it also rendered them more susceptible, apparently, to the effects of the infection. In spite of their lower parasitemia, more deaths occurred among the infected deficient chicks than among either the infected non-deficient ones or the uninfected deficient ones.

In monkeys, a deficiency of ascorbic acid modified and greatly prolonged the course of infection with *Plasmodium knowlesi*, a malarial parasite which normally produces in monkeys an acute rapidly fatal infection (32).

Instances in which a nutritional deficiency in the host lowers its resistance to protozoan parasites are somewhat more numerous than the instances, already cited, in which the reverse has been found true. Salazzo (33) found that while normal pigeons were completely refractory to infection by *Trypanosoma brucei*, a parasite of mammals, pigeons made deficient in vitamin B complex by feeding them a diet of autoclaved wheat or polished rice showed an appreciable susceptibility to this parasite. The trypanosomes became sufficiently numerous in the blood of some of these pigeons to be demonstrable microscopically as well as by subinoculation into mice.

The resistance of rats to the blood flagellate *Trypanosoma lewisi* is markedly

lowered by deficiency in either biotin or pantothenic acid (34, 35). Except in very young rats (36), *T. lewisi* is normally a benign parasite, rarely reaching densities greater than two or three hundred thousand per mm^3 of blood and never causing death. In rats fed a diet deficient in pantothenic acid and infected with *T. lewisi*, peak parasite densities were attained 5 to 9 times as high as those seen in litter mates receiving pantothenic acid (37). In the deficient animals the parasites persisted 4 to 5 days longer than in the controls, and produced a considerable anemia. It is interesting that closely parallel effects were observed in rats which received sodium salicylate (38), a compound which antagonizes pantothenic acid in the growth of certain bacteria (39).

If rats were made moderately to severely deficient in biotin by feeding them a diet rich in egg white and were then inoculated with *T. lewisi*, they showed much heavier and more prolonged infections than did control non-deficient rats (40). Even in slightly deficient rats, significantly higher parasite densities were attained than in the controls; this suggests a specific effect of the biotin deficiency. Rats which showed marked anemia as a result of having been fed a toxic low-protein diet containing 16% linoleic acid, were nevertheless fully as resistant to *T. lewisi* as rats on an adequate diet.

The first report of the effect of a specific nutritional factor on the course of a malarial infection was concerned with the influence of biotin on susceptibility to avian malaria (41). It was found that a parasitemia of approximately twice the intensity developed in chickens or ducks made moderately biotin-deficient as compared with control non-deficient birds. This was true for *Plasmodium lophurae* both in chickens and ducks and for *P. cathemerium* in ducks. These results were soon confirmed (42).

A deficiency in folic acid (43) or in protein (44), also decreases the resistance of chickens to *P. lophurae*. Since folic acid deficiency in chickens is accompanied by anemia as well as leucopenia, the higher relative parasite counts observed may have been partly due to the smaller number of red cells. Seeler and Ott (45) reported a biphasic effect of thiamine deficiency on the course of infection with *P. lophurae* in chickens. The highest peak of parasite numbers occurred in chicks with severe thiamine deficiency, the lowest in chicks receiving the minimum requirement, and intermediate peaks in those receiving an excess of thiamine. Roos, Hegsted and Stare (46) were, however, unable to find any effect of thiamine deficiency on *P. lophurae* either in chicks or in ducks. Similarly, they found no effect either of vitamin A or niacin deficiency on the course of the infection in ducks. But niacin deficiency had an interesting effect on infection with *P. lophurae* in chickens. Peak parasite densities were reached 3 to 4 times as high as those of the controls, but the birds became free from parasites at almost the same time as the controls. Brooke (47) has described effects of poor diet in decreasing the resistance of birds to avian malarial parasites. The nature of the specific deficiencies responsible for the effects observed was not determined.

The experimental study of nutrition in relation to resistance to infection can

be approached from different angles and with different ends in view. Such studies may conceivably give an insight into the influence of nutrition on the epidemiology of human disease. They may also provide a method of investigating some of the mechanisms of resistance to infectious agents. Where a nutritional deficiency lowers the extent of growth of the parasite, and at the same time appears to enhance resistance of the host as in chickens deficient in pantothenic acid and infected with *Plasmodium gallinaceum*, it seems reasonable to assume that the lack of the nutrient renders the host a relatively unsuitable culture medium for the parasite. If the nutritional deficiency produces a leucopenia, as in folic acid deficiency, it could well interfere with phagocytosis, and a deficiency which interferes with protein synthesis could also interfere with the formation of antibody globulin and hence with that important mechanism of resistance. In fact, most workers have attempted to interpret the deleterious effects of nutritional deficiency on resistance to infection in terms of interference with the formation of antibodies of the classical immunological type. This interpretation may well be correct in some instances. It is interesting to note, however, that pyridoxine deficiency, which markedly reduces the amount of lymphoid tissue (48) and the antibody producing power of the animal, has had no observed effect on resistance to primary infection with a protozoan parasite. Protein deficiency which, as Cannon (49) has shown, lowers the ability to produce antibodies, had no effect on the resistance of the albino rat to *Trypanosoma lewisi* (40). Extreme protein deficiency, of the type which reduces the resistance of chicks to *Plasmodium lophurae* (44), might well interfere with many mechanisms other than those concerned with the synthesis of antibody gamma globulins.

Becker and Gallagher (38) have suggested that pantothenic acid deficiency or the administration of salicylates reduces the resistance of rats to *Trypanosoma lewisi* because they prevent the utilization of pantothenic acid as a coenzyme in the formation of a specific oxidative enzyme which inhibits the reproduction of the parasite. This enzyme might then correspond to Taliaferro's (50) ablastin or reproduction-inhibiting antibody.

Caldwell and György (40) found that, if large numbers of mature trypanosomes (*T. lewisi*) were injected intravenously into rats, and if the rats were then injected with hyperimmune serum which had been heated to 56 C for one-half hour, the trypanosomes rapidly disappeared, if the rats had been fed an adequate diet, but they persisted and multiplied in biotin-deficient rats. These results may indicate some abnormality with respect to complement in biotin-deficient rats. However, the hemolytic complement titer of the serum of biotin-deficient chicks did not differ from that of normally fed controls (51).

The more obvious explanations for the effect of biotin deficiency in decreasing the resistance of chickens and ducks to malaria parasites have no supporting data. Moderate biotin deficiency in these birds does not affect erythrocyte or leucocyte formation, nor is there evidence that it interferes with the production of antibodies. The plasma of these birds (as well as that of man and other animals) has been found to contain a lipoprotein which on hydrolysis yields a fatty ma-

terial having biotin activity for bacteria, mosquito larvae and chicks (52). This lipoprotein occurred at a relatively high concentration in the plasma of ducks recovering from a malarial infection and at a low concentration in the plasma of ducks about to die from such an infection. The α- and β-globulin fractions of human plasma, which are rich in this material, inhibited the growth of *Plasmodium lophurae* in suspensions of duck erythrocytes *in vitro*, whereas the fractions poor in it did not have such an effect (51, 53). On the basis of this admittedly circumstantial evidence, one hypothesis assumes that a lipoprotein of the plasma at suitable concentrations inhibits the growth of malaria parasites and contributes to their destruction by the phagocytes. Biotin is essential for the synthesis of the lipoid portion of this lipoprotein, hence a biotin deficiency would interfere with resistance to malaria. Recently direct evidence has been obtained for the existence of a substance in the plasma of mature chickens (relatively resistant to *P. lophurae*) which when injected intravenously into baby chicks (highly susceptible to *P. lophurae*) reduces the severity of their infection with this parasite.

In all studies on resistance, it is important to keep in mind the different levels at which a host may oppose attack by a parasite. Frequently resistance depends on the ability of the host to prevent the entrance of the parasite into its body. This may be accomplished by simple mechanical means, or by a chemical mechanism such as the killing of the potential parasite by the acid secretion of the stomach. It may also be brought about by an extreme cellular susceptibility, as with some fungus and virus infections of plants, where the cells initially infected die so quickly that the parasite, which requires living cells to nourish it, also dies before it can progress to the infection of adjacent tissues (54, 55). Once a parasite has succeeded in entering a host, the further course of events will depend on the suitability of the host as a source of the nutrients required by the parasite and on the ability of the host to check the multiplication or growth of the parasite. This is the level of resistance which has been dealt with in most studies on resistance to protozoan infection. The protozoa, and also some of the helminth parasites are especially well suited for studies of this type, since, as Taliaferro (56) has emphasized, with these organisms one can readily follow not only the over-all rate of actual increase in numbers or of growth, but also the rate of reproduction. One does not need to depend exclusively on relatively gross criteria such as the sickness or death of the host animal. These latter criteria depend not only on the level of resistance determined by the ability of the host to check the multiplication or growth of the parasite, but also on a third level of resistance, namely the ability of the host to overcome the various injurious and toxic effects brought about by the parasite. In infections such as malaria in which the number of parasites can be determined, it is not uncommon to observe individuals with severe symptoms, but with nevertheless an appreciably lighter parasitemia than that exhibited by other individuals who show few or perhaps no signs of the disease.

NUTRITION AND SUSCEPTIBILITY TO EXPERIMENTAL BACTERIAL INFECTIONS

L. S. McClung

In considering the older literature, I wish to quote from an extensive paper by Lassen in 1931 (57) on the course of paratyphoid infections in avitaminotic rats, particularly those deficient in vitamin A. His summary, which is perhaps typical of the period, states: "... every deficiency in nutrition, whether of quantitative nature or qualitative, is accompanied by an increased susceptibility to infection and a lowered resistance." With this as a point of departure, we may examine some of the claims in recent reports to see if the same summary could be made at the present.

Higgins and Feldman (58) reported that diets significantly low in thiamine and riboflavin, inadequate to provide proper somatic growth, did not influence in any recognizable manner the resistance of the white rat to intravenous injection with virulent avian tubercle bacilli; and, an abundance of vitamin C fed to guinea pigs before and after subcutaneous inoculation of 10,000 tubercle bacilli of H37 *Rv* variant did not influence the course of the disease according to Heise and Steenken (59).

Kelly (60) reported that *Spirillum sputigenum* produced local inflammation and abscesses in guinea pigs deficient in vitamin C but was non-pathogenic for healthy animals.

Badger (61) and Badger, Masunaga and Wolfe (62) found that rats deficient in thiamine were more susceptible to rat leprosy induced by a variety of routes than were control animals on an adequate diet. Rats on the deficient diet plus supplementary purified thiamine were no more susceptible than normal rats.

Saslow, *et al.* (63a) reported that monkeys on a deficient diet developed a striking granulopenic leucopenia and showed a markedly lowered resistance to spontaneous infection with high mortality. Likewise, in contrast to controls on a normal diet, these animals showed increased susceptibility to infections with *Streptococcus hemolyticus*, Group C, and influenza virus, Type A, when these agents were administered intranasally.

Several groups have studied the problem of susceptibility or resistance to pneumococcal infection. Wooley and Sebrell (64, 65) found that Swiss mice on a diet with less than minimal growth requirements of riboflavin or thiamine are more susceptible to intranasal infection with Type I pneumococci than animals receiving suficient quantities of these vitamins for good growth. Day and McClung (66), however, found no significant change in susceptibility to intraperitoneal injection of these organisms in experimental rats showing pantothenic acid deficiency compared with the normals in the same series. With mice, similarly injected and similarly deficient, no difference in susceptibility could be observed except possibly those in acute pantothenic acid deficiency. The claim of West, *et al.* (67) that pantothenic acid deficiency greatly increased the resistance of the rat to the nasal insufflation of Type I pneumococcus must be viewed with some care since only 8 animals were used. Robinson and Siegel (68)

conducted more extensive studies, also with Type I pneumococci in rats, employing 30 to 50 viable cells in a broth-mucin suspension and the intratracheal route through an incision in the neck. They reported that rats deficient in riboflavin or pantothenic acid are as resistant to experimentally induced pneumococcal lobar pneumonia as those receiving the same basal diet with adequate quantities of these factors, but that thiamine and possibly pyridoxine deficiency appeared to lower the resistance. These authors also considered the vitamin content of the tissue of the depleted animals and found sufficient quantities of the vitamins to support the growth of the microörganism. In a later report, Hitchings and Falco (69) discovered that mice on a purified diet survived the intraperitoneal injection of 100,000 lethal doses as compared with those on a commercial laboratory diet. They were unable to ascribe the difference to any known dietary essential.

With respect to infection by the salmonella, which are natural pathogens for mice, Stryker and Janeta (70) found no noteworthy difference in intestinal permeability to *Salmonella enteritidis* or of *Clostridium botulinum* toxin in rats deficient in vitamin A and control animals. Riboflavin deficient mice are much more highly susceptible to spontaneous salmonella infections than control animals according to Kligler, *et al.* (71), and likewise mice and rats in a state of avitaminosis A are more susceptible than normals (72) but with this factor included in the diet. Paired feeding experiments suggest that starvation rather than vitamin A deficiency was the more important factor. Biotin deficient rats, which were infected in groups after 14, 21, and 28 days on the diet by feeding a low dilution of a culture of *S. typhimurium* and were killed 3 days later, showed greater percentage of positive cultures of *Salmonella* from their organs than did controls. In spontaneous salmonella epidemics in mice, employing 4 week old litter mates in the groups, the deficient animals showed a higher rate of infection (73). With respect to thiamine, Guggenheim and Buechler (74) reported that deficient mice showed a markedly increased susceptibility to oral infection with *S. typhimurium* and it was suggested that with mice the diminished resistance is exclusively attributable to the thiamine deficiency, whereas the higher susceptibility of rats is due to the inanition which accompanied avitaminosis.

This short summary of claims in recent reports seems to fail to support completely the statements of Lassen given previously. In addition, in order to keep this summary within appropriate limits, we have refrained from pointing out, for each report, factors which might cast doubt on the validity of the conclusions suggested by the authors. In many, if not all, of the reports, serious criticism could be made of one or more of the items in the experimental procedures used.

INFLUENCE OF NUTRITION IN RESISTANCE TO EXPERIMENTAL RICKETTSIAL INFECTION

Henry Pinkerton

Historically, high typhus mortality has through the centuries been associated with war and famine, and on the experimental side, certain dietary factors have

influenced markedly the response to rickettsial infection. Our experience with rickettsial infections has taught us that it may be difficult to determine whether certain compounds alter resistance by affecting the nutrition of the host or that of the invading organisms. We shall, however, consider primarily the nutrition of the host, since specific information about the nutrition of the rickettsiae is largely lacking. Our statements will be confined chiefly to typhus infection, but diseases of the spotted fever and tsutsugamushi groups appear to react in a similar manner to nutritional alterations.

Growth Requirements of the Rickettsiae. The rickettsiae are obligate intracellular parasites and have been cultivated only on this basis. Wolbach and his co-workers (81), in 1923, obtained slight multiplication of spotted fever and typhus rickettsiae in plasma tissue cultures maintained at 37.5 C. In 1931, Nigg and Landsteiner grew typhus rickettsiae in large numbers within cells in a modified Maitland culture (minced guinea pig tissue in a mixture of serum and Tyrode's solution) incubated at 37.5 C. In the same year, Pinkerton and Hass repeated the earlier attempts of Wolbach and his co-workers to grow rickettsiae within living cells in plasma tissue cultures. At 37.5 C, rickettsial multiplication was slight and transient, whereas at 32 C, massive intracellular growth of rickettsiae occured (83), and heavily infected cultures could be maintained indefinitely (84). In the Maitland type of culture, tissue cells do not multiply, but slowly die, and probably for this reason the temperature of incubation was not so important. The evidence suggested that rickettsiae did not prefer a temperature of 32 C, but grew freely in living cells maintained at that temperature because the lowered metabolic rate favored their multiplication.

This concept received more direct experimental support by the work of Zinsser and Schoenbach (85) in 1937. These authors found that in cultures of the Maitland type, incubated at 37.5 C, rickettsial growth was most active in the sixth, seventh, and eighth days of incubation, "when the tissue has ceased respiring, and is either not viable at all, or has lost much, possibly all, of its metabolic activity." This result was in striking contrast to that obtained with a typical virus (equine encephalitis) which multiplied most actively during the first two days of incubation, when tissue respiration was most active.

The oxygen tension and pH of plasma tissue cultures did not influence the growth of rickettsiae unless the alteration was sufficient to cause the death of the host cells (84). The rickettsiae of spotted fever in tissue culture, for some unknown reason, grew most freely within the nuclei of their host cells, where they formed compact clusters somewhat resembling the nuclear inclusions of viral infections (86). Typhus rickettsiae were never seen in nuclei; they were reduced in number and spherical in shape in cells which were dividing mitotically, again suggesting that an increase in cellular metabolism was unfavorable for rickettsial growth.

Zinsser and his co-workers (87) improved the Maitland medium by using Tyrode's solution with serum agar, and by spreading the minced tissue on the surface of this medium. Zia (88) grew rickettsiae on the chorioallantoic membrane of the fertile egg; and Cox (89) grew them even more abundantly in the

yolk sac. In all of these media, rickettsiae grow only within cells although, after the host cells die, many extracellular rickettsiae are found. The peculiar susceptibility of the cells lining the yolk sac of the fertile egg has not been explained. These cells are most susceptible on the fifth and sixth days of embryonic development; active multiplication takes place at temperatures between 34 and 37.5 C, and very little growth of rickettsiae occurs if the eggs are incubated at 40 C (90).

It may be assumed that rickettsiae are at least partially lacking in vital enzyme systems, and can metabolize only by diverting to their own use certain enzyme systems of the host cells. This nutritional dependence of rickettsiae on the host cells suggests that agents which modify the intracellular metabolism in the host might affect, favorably or unfavorably, the resistance of the host to infection.

Genetic Factors. Experimental typhus is normally a mild, nonfatal disease in the guinea pig and in the rat, but is fatal for certain strains of mice. The variable susceptibility of different strains of mice (91) and of fertile eggs (92) indicates again the importance of genetic differences stressed in the first section of this review.

Starvation. We have studied the effects of starvation on murine typhus infection in guinea pigs and rats (93). In male guinea pigs on a normal diet, such as oats, alfalfa, carrots, and water, the intraperitoneal injection of rickettsiae causes, after an incubation period of two to four days, marked inflammation of the scrotal sac, redness and swelling of the scrotum, and a febrile reaction lasting for several days, although the animals do not appear ill, and invariably recover. A fibrinous exudate, containing small to moderate numbers of rickettsiae is found in the scrotal sac, where the temperature is low (about 32 C), but the general peritoneal cavity remains normal. If male guinea pigs are deprived of food (but given water *ad lib.*) from the day of injection, neither scrotal reaction nor fever occur. Typhus-infected male guinea pigs which are killed after five to eight days of starvation, however, show a heavily infected gelatinous exudate in the general peritoneal cavity as well as in the scrotal sac. In starving rats injected intraperitoneally with murine typhus rickettsiae, we also found a copious exudate and a much greater accumulation of rickettsiae in the peritoneal cavity than in the control rats fed on dog chow and water.

Intoxication by benzol or by X-rays according to Zinsser and his co-workers (94) greatly reduces the resistance of rats to murine typhus rickettsiae, and causes the accumulation of rickettsiae in great numbers in the peritoneal cavity, where normally only a few rickettsiae were found. These methods, particularly the irradiation method, were used extensively to obtain rickettsiae in large numbers for carrying out immunological studies.

Vitamins. In 1931, Zinsser and his associates (95) found that a diet lacking all of the then known vitamins, carried to a point where deficiency signs developed, increased the severity of murine typhus infection in guinea pigs and rats, and caused the appearance of a pleural and peritoneal exudate with many rickettsiae. The effect which they got in guinea pigs was probably the result of ascorbic acid deficiency.

In 1939, Pinkerton and Bessey (96) reported a striking and apparently specific effect of riboflavin deficiency on experimental murine typhus infection in rats. Even in the early stages of deficiency when the rats were in relatively good condition (and had a life expectancy of three to four weeks), a complete loss of resistance was found, and the rats died in three to four days with an overwhelming infection, characterized by huge numbers of rickettsiae in all of their organs. The administration of riboflavin to such rats, even when they were at the point of death, caused complete recovery within twenty-four hours.

Vitamin A deficiency in rats, even in the late stages, did not cause appreciable loss of resistance to murine typhus (96).

Oral administration of para-aminobenzoic acid (PABA) to mice, by Snyder and his co-workers (97) and independently by Greiff and Pinkerton (92), was found strikingly to reduce the mortality from murine typhus. It is effective also against experimental spotted fever and tsutsugamushi disease. Although PABA forms part of the folic acid molecule, folic acid itself is not rickettsiostatic in the fertile egg (98).

We have injected many vitamins into typhus-infected embryonate eggs without definite effects, but it is probable that egg yolk contains adequate amounts of most vitamins. Generally speaking, vitamins are ineffective unless a deficiency exists. PABA is an exception to this statement, but it should be noted that the quantities of PABA needed to cause rickettsiostasis are enormous in comparison with the amounts required to maintain health. Recently developed knowledge of antivitamins and anti-metabolites may well prove useful in solving problems of rickettsial and viral metabolism.

Fitzpatrick (99) has found recently that rats showed an increased susceptibility to murine typhus when kept on diets deficient in the following specific ways: (*a*) low proteins; (*b*) one-tenth of the optimum supply of all vitamins of the B group; (*c*) one-twentieth of the optimum supply of pantothenic acid; (*d*) one-twentieth of the optimum supply of riboflavin; (*e*) one-twentieth of the optimum amount of thiamine. No change in susceptibility was found in rats kept on diets deficient only in containing one-fortieth of the optimum amount of pyridoxine, choline, nicotinic acid, and para-aminobenzoic acid. Susceptibility was measured by the mortality, which was usually zero in the rats on a complete diet and often 100 percent in deficient animals.

The addition of one per cent of liver powder to a complete diet did not increase resistance, but rats on a natural laboratory diet (oats, Rockland diet and fresh vegetables) were less susceptible to infection than those kept on a complete synthetic diet, even though the latter caused a greater increase in weight.

We have recently found that certain folic acid antagonists and derivatives (gamopterin, diopterin, aminopterin, and pteropterin) in maximum tolerated doses have no appreciable effect on rickettsial growth in fertile eggs (100). These compounds were injected forty-eight hours after the injection of rickettsiae.

Dyes. Methylene blue and toluidine blue, when incorporated in the diet, increase resistance and greatly lower the mortality from typhus infection in mice (101). Unfortunately, the toxicity of these drugs has made them unsuitable for use in human beings.

Other Chemotherapeutic Agents. The sulfonamides lower resistance to experimental spotted fever and typhus infection in guinea pigs (102, 103). Penicillin is rickettsiostatic against murine typhus infection in the fertile egg (104), and reduces the mortality of mice from murine typhus infection (105) from 100 percent to zero if given early, under certain experimental conditions. Parasulphonamidobenzamidine and related compounds are effective in reducing the mortality from typhus infection in mice (106). Acridine compounds are rickettsiostatic in the fertile egg (107).

Chloromycetin has been shown recently (108) to be remarkably effective against experimental infection with the rickettsiae of typhus, spotted fever, tsutsugamushi disease, and rickettsial pox. It is effective when given either orally or intraperitoneally to mice infected with tsutsugamushi disease. This compound is also effective against psittacosis. Although psittacosis is classed as a viral disease, it is biologically more closely related to the rickettsiae than to the smaller and more typical viruses.

Aureomycin (109) also is effective against spotted fever, tsutsugamushi disease, and Q-fever, both in fertile eggs and in experimental animals.

Para-aminobenzoic acid is probably ineffective against Q-fever, but streptomycin is said to be of value (110). Q-fever differs rather widely from other rickettsial infections.

Mechanisms of Action. In attempting to explain the bacteriostatic action of chemotherapeutic agents, emphasis has been placed on interference with the nutrition of the invading organisms. The intracellular location of the rickettsiae probably protects them somewhat against the direct action of most chemotherapeutic agents. The rickettsiae probably are vulnerable for a short time while they are moving from one cell to another, however, and certain rickettsiostatic agents, such as penicillin, aureomycin, and chloromycetin, may inhibit rickettsial multiplication directly in much the same way as they inhibit bacterial multiplication. We have also indications for believing that certain rickettsiostatic agents may act indirectly by altering the metabolism of the cells in which the rickettsiae multiply.

Smadel and his associates have suggested, on the basis of their observation that nucleic acid partially neutralized the action of the acridine dyes in the fertile egg, that the rickettsiostatic action of these compounds may depend on interference with an adenine-containing enzyme of the organism or host cell (107).

Lowering the temperature of infected animals is known to favor the intracellular growth of rickettsiae and the scrotal reaction, which is characteristic of many rickettsial infections in experimental animals, is undoubtedly due to the relatively low temperature of the scrotum. Typhus-infected mice, kept at a temperature of 29 to 37 C showed a survival rate of ninety percent, as compared with a survival rate of zero in parallel series of mice kept at 18 to 22 C (111).

This temperature effect makes it difficult to interpret the results of many of the experiments cited. The rapid multiplication of rickettsiae in the peritoneal

cavities of animals made ill by starvation, benzol or X-ray intoxication, and certain vitamin deficiencies may depend, to some extent at least, on a non-specific lowering of the body temperature. Zinsser and his co-workers were inclined to attribute the effect of benzol and irradiation to the destructive action of these agents on hematopoietic tissue, but this point was not proved.

The mechanism of action of temperature changes in modifying rickettsial multiplication is not clear. Phagocytosis of staphylococci by guinea pig leucocytes (112) increases with rising temperature to a maximum at 40 C, and this phenomenon may be of some importance in rickettsial infection. The importance of a low temperature (32 C) in obtaining vigorous intracellular multiplication of rickettsiae in plasma tissue cultures (83), where leucocytes are not present, suggests that temperature changes may affect rickettsial growth principally by altering intracellular metabolism.

Pinkerton and Bessey (96) believed that the loss of resistance to murine typhus resulting from riboflavin deficiency might be a direct result of lowered intracellular respiration, since riboflavin forms an essential component of an important respiratory enzyme (113).

One might be tempted to explain the rickettsiostatic action of such dyes as methylene blue, toluidine blue, and nitroacridine on the basis of their known ability to increase oxygen consumption *in vitro* (114), although it has not been shown that they have a similar effect *in vivo*.

Greiff and Pinkerton (98) have attempted to determine to what extent the rate of cellular respiration (as measured by oxygen uptake) could be correlated with rickettsial multiplication in the yolk sac of the fertile egg. An increase in the environmental temperature from 37.5 C to 40 C, which is accompanied by an increased oxygen consumption, almost completely inhibited rickettsial growth. When KCN was injected into the yolk sac, in amounts insufficient to kill the embryos, rickettsiae multiplied freely at 40 C. KCN lowered the oxygen consumption of the eggs and under certain conditions enhanced greatly the multiplication of rickettsiae. PABA, which is markedly rickettsiostatic, increased the oxygen consumption in eggs about 50%. Ortho-aminobenzoic acid and meta-aminobenzoic acid, which were not rickettsiostatic, did not increase the oxygen consumption in eggs. Under certain experimental conditions, therefore, rickettsial growth appears to be inversely proportional to cellular respiration.

The relation of metabolic processes, other than respiration, to rickettsial multiplication has not been studied. I know of no instance in which dietary deficiency has caused increased resistance to rickettsial infection, and in this respect the rickettsiae appear to differ from certain bacteria, protozoa, and viruses.

In conclusion, although only a few preliminary steps have been taken, the study of the influence of nutrition on resistance to rickettsial infection has already led to results of great interest and importance. At the present time wide gaps exist in the information which biochemists can give us regarding intracellular metabolism, and even wider gaps between the detailed knowledge of certain enzyme systems and the application of such knowledge to problems of intracellular parasitism.

THE EFFECT OF NUTRITIONAL FACTORS ON THE RELATIONSHIP BETWEEN THE BACTERIAL VIRUS AND ITS HOST

Winston H. Price

This section of the review is concerned with work done between 1943 and 1948 on the effect of various nutritional factors on the formation of bacterial viruses. It will become apparent that the relationship between a bacterial virus and its host is not a static one, but, on the contrary, is a dynamic system in which various nutrients determine the interaction between the bacterium and its virus.

Before examining the effect of various nutritional factors on bacterial virus systems, it will be profitable to state briefly the cycle of growth of the virus and the characteristics of a desirable system for studying nutritional effects. The growth cycle of the virus occurs in three steps (2): (*a*) the adsorption of the virus on the host cell, (*b*) the multiplication of the virus and (*c*) the release of the virus from the cell. During step *b*, which is called the minimum latent period, no increase in virus titer as measured by plaque counts has been demonstrated, since this method determines only extracellular virus. The virus particles are released from the cell at a time characteristic for a given virus; this produces a definite increase in virus titer. By using the one-step growth technique of Delbrück and Luria (115), one may determine whether the nutrients are involved in the adsorption of the virus to the host, or in the actual multiplication of virus, or whether the metabolite is concerned primarily with the lytic process.

Experimental evidence indicates that bacteriophage multiplication requires certain cellular reactions, but not necessarily all that are essential for bacterial reproduction (116). An appropriate system to study the effect of nutrients on virus growth would be one in which cellular multiplication did not contribute substrates for virus formation; therefore, a non-infected cell incapable of growth in the culture medium used would be an ideal host for such an investigation. Five such systems have been reported. Spizizen (116) found that cells of *Escherichia coli* suspended in a solution of 17.4×10^{-4} M glycine anhydride would support the growth of phage, although this medium would not support bacterial multiplication. Experiments by Price (117) and Krueger and co-workers (118) have indicated that staphylococcus cells would form phage under conditions where penicillin prevented any demonstrable multiplication of the cells. Herriott and Price (119) have shown that cells of *E. coli* and staphylococcus rendered nonviable by mustard gas would form phage. Finally, Anderson (120) has reported that cells of *E. coli* supported phage formation after ultraviolet irradiation although the cells were no longer capable of multiplication.

It is desirable to study those nutrients which affect virus reproduction but not the multiplication of normal cells, for if the latter is affected, it is more difficult to determine the mode of action of the nutrient. Finally, it is easier to interpret nutrient requirements and relationships in a cell which does not have too varied synthetic capacities. It should be pointed out that although an added nutrient may have no observable effect on virus formation, this nutrient substance may

be concerned in virus reproduction. The lack of effect of the nutrient may be due to the fact that the substance is synthesized by the host at a rate fast enough so as not to be a limiting substrate in virus synthesis.

The Effect of Amino Acids. Amino acids influence the formation of bacterial viruses in several interesting ways. Anderson (120) has carried out an extensive study on the effect of various amino acids on the adsorption of the virus to the host cell. He has shown that viruses T4 and T6 of *E. coli* require tryptophan for this process, that the concentration of tryptophan, temperature, pH, and length of exposure are factors in the adsorption and that the process is reversible by dilution in a synthetic medium. The degree of cofactor requirement appears to be inherited in clones of T4. Anderson has also presented evidence that coli virus T1 needs isoleucine, norleucine or methionine for adsorption, and coli virus T7, leucine, isoleucine or methionine. Although the mechanism whereby the virus becomes attached to the host cell is unknown, the finding of Anderson that certain amino acids are necessary to activate the virus before adsorption takes place may indicate that an enzyme reaction is involved in this process (120).

Cohen and Fowler (121) have found that amino acids may play a role in the formation of bacterial viruses other than a requirement for the adsorption process. Studying the formation of coli virus $T2_{r+}$ in a synthetic medium consisting of salts, ammonium lactate and ammonium chloride, they observed that the yield of virus per cell was much lower and the minimum latent period longer than on cells grown in nutrient broth. The combination of valine, isoleucine, leucine, phenylalanine, histidine, arginine, lysine, aspartic acid, glutamic acid, methionine, tryptophan, tyrosine and the purine, adenine, when added to the synthetic medium gave a burst size 90% of that found in broth and a latent period lasting 1 to 2 minutes longer. They also observed that certain amino acids decreased the minimum latent period with little effect on the yield of virus (122). For example, proline shortened the minimum latent period to a greater extent than aspartic acid, although the phage yield per cell was higher with aspartic acid. These experiments indicate that the time of liberation of the virus seems to be independent of the amount of virus synthesized.

Leucine inhibits virus reproduction, and this inhibition was overcome by the addition of valine, isoleucine or norleucine; in fact, the virus increase was greater when this inhibition was overcome than with the latter three amino acids alone. Alanine, threonine, methionine, and tryptophan had no observable effect on virus proliferation.

Spizizen (116) has reported interesting observations on coli viruses grown in glycine medium. The interpretation of these experiments is complicated by the fact that the virus-infected cells were incubated seven minutes in broth before being added to the glycine solution. Using this technique, however, he found that cells suspended in a solution of 19.5×10^{-4} M glycine or 17.4×10^{-4} M glycine anhydride were not capable of multiplication although they supported virus reproduction. Moreover, 36×10^{-4} M aminomethane sulfonic acid, the sulfonic acid analogue of glycine, inhibited virus formation in a 13×10^{-4} M glycine solution but not in a 6.5×10^{-4} M xanthine solution or in nutrient broth. Spizizen also found

that when cells of *E. coli* were kept in a solution of 13×10^{-4} M glycine for 16 days at 37 C with a trace of nutrient broth, and then transferred to a fresh 13×10^{-4} M glycine solution, they gave fairly high increases in virus. If these cells were transferred to broth, however, there was very little virus reproduction. On the other hand, cells kept first in broth for 16 days at 37 C and then transferred to a 13×10^{-4} M glycine solution formed little virus, although when these cells were added to nutrient broth a fairly large increase in virus was observed. While these latter results are difficult to interpret, they indicate how the nutritional state of the host *previous* to the infection may determine the effect of a nutrient on subsequent bacteriophage formation.

The Effect of Other Nutrients. Spizizen (123) has reported the effect of various nutrients on the formation of coli virus. The addition of yeast nucleic acid, adenosine triphosphate, glycerophosphate, diphosphopyridine, glucose-6-phosphate, and adenylic acid to a solution containing 8.7×10^{-4} M glycine stimulated bacteriophage multiplication. However, not all phosphorylated compounds were stimulatory, since fructose-6-diphosphate, glucose-1-phosphate, fructose-6-phosphate and guanylic acid were inactive. Calcium pantothentate, thiamine, nicotinic acid and pyridoxine had no phage stimulating action in the glycine medium.

Fowler and Cohen (122) have shown that an external source of carbon, nitrogen and phosphorus is essential for the reproduction of $T2_r +$ virus of *E. coli*. Cohen has interpreted his experiments to mean that in a virus-infected cell only virus material is synthesized (124). While this interpretation may be correct, it is not yet definitely established.

Experiments from this laboratory indicate that certain nondialyzable fractions from *Staphylococcus muscae* and yeast will increase the phage yield per cell of *S. muscae* in Fildes' synthetic medium containing hydrolyzed casein (125). If the cells were grown in the presence of the fraction and then centrifuged out, washed, and suspended in fresh Fildes' synthetic medium plus hydrolyzed casein, such bacteria showed an increased phage yield, if virus was added immediately (126). The active substance therefore seems to be removed from the medium by the cells. If cells previously treated with the yeast fraction were suspended in fresh synthetic medium free of this fraction and allowed first to incubate one hour, and virus then added, there was no increased phage yield. This experiment indicates that in the absence of virus, the cells convert the substance to a form not utilizable in virus synthesis. These fractions have a high concentration of ribonucleoprotein which increases as the fractions become purer. The yeast fraction isolated by Reiner and Spiegelman (127) which stimulates adaptive enzyme formation in yeast can also stimulate virus formation in the *S. muscae* system. The method of isolation and the properties of the substance isolated in both laboratories are very similar. Whether the active substance is a ribonucleoprotein and whether the two substances are the same must await the purification of each compound.

Cellular Metabolic Patterns Determining Host Resistance. In recent years, three

virus systems have been reported in which the host metabolic pattern has made them resistant to virus action.

E. H. Anderson (126) has reported that a single spontaneous mutation in *E. coli* resulted in a mutant resistant to the virus. This mutant differed from the wild type in requiring tryptophan to grow on synthetic medium consisting of inorganic salts, dextrose and ammonium chloride, and also in growing much better when a source of amino nitrogen such as asparagine was added to the medium. The mutant was resistant to T1 but not to T5. Mutants resistant to T1 and T5 and capable of growth with inorganic nitrogen and glucose were also uncovered. Another resistant mutant was isolated which required for growth both tryptophan and proline. It is not yet established whether these specific metabolic changes are directly correlated with virus resistance.

Studying various strains of virus T4 of *E. coli*, Delbrück has found that one strain required tryptophan and another strain tryptophan and calcium for reproduction. The multiplication of these virus strains is inhibited by small amounts of indole. Since the host forms indole from tryptophan, virus reproduction stops when tryptophan is added to the medium (128).

Experiments from this laboratory indicate a competition between the virus and host for nutrients. Using a staphylococcus phage, we showed that a substance present in certain acid hydrolyzed proteins was needed for virus reproduction. This factor is essential for the multiplication of the virus but not for the host, although the host may remove it from the medium. It is not needed for the adsorption of the virus to the cell. Using a high initial concentration of bacteria, a low virus concentration and a small amount of the factor in Fildes' synthetic medium, the virus will multiply for a time and then stop, although the cells, since they are not all infected, keep on multiplying. This cessation of virus reproduction results from the removal of the factor from the medium by the bacteria. Addition of more factor will result in the resumption of virus reproduction (129). Preliminary evidence of a similar competitive effect has also been observed with nicotinic acid and the nucleoprotein fraction, mentioned previously, for this staphylococcus system. Such competitive systems may play some part in the detrimental effect of the virus on the cell. If the virus, by some inhibitory mechanism, prevents the utilization of essential nutrients by the cell and utilizes these nutrients for its own synthesis, the cell will eventually die. As was pointed out earlier, Cohen (124) has also presented evidence for this view.

Influence of Nutrients on the Response of the Host to Infection. Two systems have been investigated in which nutrients have modified the normal "pathological response" of the host cells to the bacterial virus. Wahl (130) found that virus of *E. coli* needed calcium to reproduce in a synthetic medium containing ammonium sulfate, potassium chloride, magnesium sulfate, inorganic phosphate and 1 μg of thiamine per ml. Under such conditions, the host cells did not lyse. The addition of 1 μg more of thiamine per ml to the medium caused cellular lysis although the virus titer remained the same. Experiments from this laboratory have shown that with *S. muscae*, phage release is correlated with cellular lysis in nutrient broth, although in Fildes' synthetic medium the virus is released without

observable lysis (131). This latter observation has been confirmed not only by turbidity measurements but also by microscopic study. The addition of a small amount of nutrient broth to the synthetic medium resulted in cellular lysis together with the release of the virus. Cellular lysis eventually takes place in Fildes' synthetic medium, but after the virus is released from the cell. For example, in veal infusion medium, cells of *S. muscae* begin to lyse 30 to 45 minutes after infection and virus begins to be released into the medium. In Fildes' synthetic medium the release of virus from the infected cells begins after 50 to 60 minutes and the maximum phage titer is reached at about 85 minutes; the cells begin to lyse in from 90 to 110 minutes.

A further analysis of the release of virus from cells of *S. muscae* grown in Fildes' synthetic medium has recently been carried out (132). The addition of a nondialyzable fraction from yeast, resulted in a correlation between cell lysis and virus release, although the minimum latent period was the same as in the system in which the virus was released without observable lysis; that is, about 40 to 50 minutes. It should be pointed out that in all these experiments the multiplicity of infection was about 1.3. If the cells were infected with more virus particles to give a multiplicity of infection of about 6, then the cells began to lyse 40 to 50 minutes after infection and virus release was correlated with cellular lysis. In all three instances the phage yield per cell was the same. This experiment shows quite clearly how the lysis of the cells may be modified by environmental conditions and indicates that cellular lysis is an accessory phenomenon associated with bacteriophage formation.

Rather similar effects of nutritional factors have been reported with animal viruses. Thus, the Wisconsin group (133) showed that in mice with a thiamine deficiency, western equine encephalomyelitis virus multiplied and killed the host, although the animals did not show the characteristic signs of this infection. This result is quite similar to the result obtained in synthetic medium with the *S. muscae* virus system where the virus killed the bacteria and multiplied, but in which the response of the host was different from that observed in a rich veal infusion medium. Toomey, *et al.* (132) also found that mice on a thiamine deficient diet injected with the Lansing virus, died without showing paralysis. The result of these experiments may indicate that a rather specific nutritional environment is necessary for the so-called "normal" response of a host to a virus infection.

THE INFLUENCE OF NUTRITION ON EXPERIMENTAL VIRUS INFECTION

Paul F. Clark

In the field of experimental virus infection, Peyton Rous (134) made one of the earliest observations on the effect of nutrition on the transmissible chicken sarcoma. He reported that intercurrent illness of the host checked the development of the tumor and that young healthy well nourished fowl proved more susceptible than the thin and the ill.

Olitsky (135) noticed a similar response in guinea pigs inoculated with the virus of foot-and-mouth disease. Frequently the animals suffering from malnutrition or intercurrent infection showed a delayed appearance of the primary and secondary vesicles. No protective antibodies were demonstrable in the blood serum of these animals so an explanation should be sought along other lines.

Rivers (136) stated that it was a common observation in his laboratory that unhealthy or malnourished rabbits showed less reaction to vaccine virus and exhibited a lower titer to the active agent than did healthy animals.

In a series of important papers, Sabin (138) and Olitsky (139) have stressed the role of constitutional barriers which develop normally as animals pass from infancy to maturity. They have studied these changes with several viruses especially those of vesicular stomatitis and equine encephalomyelitis in mice. Several peripheral routes of infection such as nose, peritoneal cavity, muscle, or skin are readily effective in young mice and completely or largely ineffective in adult animals, depending on their age and the choice of routes. During maturation, barriers, quite unrelated to humoral immunity, develop which block the routes of dissemination possible in young animals. They have localized a number of these barriers with reasonable accuracy.

Sabin (140) has followed this line of query critically by modifying the maternal diets during the lactation period and also by weaning mice prematurely and raising these animals on selected diets. Either relative inanition or the absence from the diet of certain specific substances, such as thiamine and riboflavin during the period of rapid growth, will prevent or retard the appearance of some of the natural barriers to involvement of the nervous system. Further study of these "barriers", which quite likely may be chemical in nature, by the use of the recently developed histochemical methods might lead to further unravelling of the enigma. One is reminded of the wide variety of interference phenomena especially those induced by the vitamin analogues (Woolley, 141).

Other attempts to study host-cell virus relationships by several methods including alteration of the diet, may be seen in the experiments of Sprunt (142). After finding that under usual experimental conditions the volume of vaccinia virus injected intradermally is more important than the number of virus particles in determining infection, he carried out three types of experiments: (*a*) varying the amount of inoculum, (*b*) localizing the virus by injecting estrogenic hormones, and (*c*) increasing the spread of a constant quantity of virus by injecting the testicular spreading factor. These experiments indicated that when the virus was well localized and fewer host cells involved, the lesions were fewer.

In further studies, group *a* received no food but had free access to water, group *b* received neither food nor water, and group *c* received no food but had access to water and received in addition intraperitoneal injections of 50 ml physiologic saline solution twice daily. These regimes were maintained for ten days before virus inoculation and two days thereafter. The animals were then fed amounts sufficient to maintain weight, and group *b* was given free access to water. Plasma proteins showed no change throughout the experiments.

The animals were sacrificed seven days after vaccination and the following observations made:

1. Starvation but with access to water (group *a*) causes fewer or smaller vaccinia lesions than in controls.
2. Starvation plus dehydration (group *b*) causes even more marked decrease in vaccine lesions.
3. Starvation with increased interstitial fluid (group *c*) increases the number and size of lesions.

Sprunt suggests that the greater inhibition in group *b* may be due to the restriction of the spread of virus particles by dehydration so that fewer host cells are exposed while increased interstitial fluid as in group *c* has the opposite effect. It would seem more likely that some of the violent changes that occur during alterations in water balance are responsible for changes in cell permeability and therefore cell susceptibility.

Olitsky and Schlesinger (139) also stressed the factor of edema when they observed that hypertonic salt solution injected into the base of the tail of albino mice 3½ to 24 hours before the cutaneous injection of herpes virus, increased infection both on the basis of success with one hundred fold greater dilution of the virus and also shortening of the incubation period by one-third. Mixtures of hypertonic solution plus virus, injection of the hypertonic solution after the virus, and application of virus after the edema had subsided showed no enhancing effect. They suggested that the factors in edema which dilate the lymph vessels, render their walls more permeable, and increase the rate of flow, may bring the virus into contact with a greater number of nerve fibers in the corium and therefore aid infection.

Because of Heine's original observation, supported by several observers since his time, that poliomyelitis seems to attack the better nourished children with relatively higher incidence than the scrawny and ill fed, several experimental studies seeking knowledge in this field have been undertaken with the further hope of an increase in general information about host-cell virus relationships.

Experiments with Central Nervous System Viruses Transmissible to Mice. Foster (143) and her colleagues at Pennsylvania have, in a series of well-controlled experiments with Lansing strain poliomyelitis virus injected intracerebrally in mice, shown that a thiamine deficient diet prolongs the incubation, reduces the incidence of paralysis, and also the mortality rate, as compared with control groups on an unrestricted complete diet. Querying further the role of reduced food intake and inanition incident to thiamine deficiency, they report that the incidence both of paralysis and of death was less in the vitamin deficient group than in a paired group on a simple restricted diet with adequate thiamine. Both caloric and total restrictions were included in their experiments. They conclude that "apparently the effect of vitamin B_1 deficiency on the action of the virus is not due solely to anorexia."

A University of Wisconsin group of investigators made similar observations with essentially the same conclusions (Rasmussen *et al.*, 144). They have made several basic studies of the nutrition of the rhesus monkey, and have used a

wider variety of viruses of the central nervous system, not only Lansing but Theiler's GDVII, TO, and FA, western equine, all in mice, the virus of avian encephalomyelitis in chickens, as well as several strains of human poliomyelitis virus in monkeys (Clark *et al.*, 151), all these with a variety of diets.

In their thiamine deficiency experiments, they noted that after the usual period of observation (28 to 35 days with the polio and Theiler's viruses) when essentially all of the optimum fed controls had become paralyzed and had died, if the survivors on the thiamine deficient diet were given liberal thiamine, a number of these survivors (4 of 14 in a typical Lansing series) came down with typical paralysis. Apparently the virus had not been destroyed, but some interference had occurred so that the typical course of the disease was altered.

Also with another virus, western equine encephalitis, most mice with a severe thiamine deficiency (17 to 19 days on the deficient diet before inoculation) failed to show the characteristic signs of infection. Such mice showed only weakness progressing to atonia, tremors, and marked ataxia; the time of death was delayed somewhat but was earlier than in uninoculated deficient groups (147). Titration of brain-cord suspensions from these inoculated deficient animals, without any typical signs, showed that in this instance also the virus was not destroyed, but had multiplied apparently as well in these animals as in those that had shown typical encephalitis while fed on optimum diets. Characteristic brain lesions were also present both in the atypical deficient animals and in those with typical encephalitis fed a complete ration *ad libitum*. These histologic studies were not, however, carried out with detail adequate for a more definitive statement.

A few only of the other B group of vitamins have caused less marked alterations in the course of the disease. Pantothenic acid deficiency is especially interesting in that it gave lowered incidence of typical paralysis in the mice challenged with Theiler's GDVII, but no difference in similarly deficient and control groups when Lansing strain was employed. This suggests a difference in the requirements of the two viruses. The results with riboflavin deficiency were just the reverse, with no difference in the Theiler's FA and GDVII groups and a very slight statistical difference with Lansing virus. Through the use of a complete synthetic diet and carefully calculated salt mixtures, Lichstein (145) and his associates studied the effect of single mineral deficiencies on the susceptibility of Swiss mice to Theiler's GDVII virus. No demonstrable effect on the course of the disease was obtained by varying the level of calcium, magnesium or chlorine in the diet; a sodium deficiency, however, resulted in a small decrease in the number of paralyses; a progressively decreasing incidence of paralysis was observed as the amount of potassium or phosphorus was decreased from optimum to an essentially completely deficient level.

It should be noted, however, that although the incidence of paralysis varied from 14 to 37% in the K deficient groups as compared with 78 to 95% in K optimum groups, deaths without paralysis invariably brought the total fatalities to 100% in inoculated mice at a time when very few uninoculated deficient mice had died. This effect should probably, therefore, be designated as an alteration in the clinical picture rather than a definite increase in resistance.

Digging further into the proteins, we have employed acid-hydrolyzed casein,

with the essential amino acids in purified form, deleting one after another of the amino acids from the otherwise complete diet. The tryptophan deficient diet has given the most marked results (Kearney *et al.*, 148) with a deficiency in valine or isoleucine providing similar though less marked alterations in the clinical picture. The mice fed an optimum diet minus tryptophan showed an accelerated death rate in those inoculated with Theiler's GDVII as compared with the control deficients and a marked lack of the characteristic signs of the disease, commonly almost no cases of paralysis, as contrasted with close to 100% in groups on optimum diets. Yet in these tryptophan deficient mice, showing no signs of paralysis, in fact some of them with no signs of virus infection, the virus had multiplied abundantly as determined by subsequent brain-cord titration. The proliferation of the virus seems to have been equal to that in the optimum fed typically paralyzed animals, although more complete titrations of separate portions of brain and cord would be needed to support this statement adequately.

Many of these experiments show clearly and with reiterated emphasis that the typical clinical picture of several virus diseases can be markedly altered by a variety of dietary deficiencies. This alteration extends even to the elimination of paralysis in a high percentage of the cases but, in these altered or resistant mice, the virus continues to multiply apparently as abundantly as in animals fed a complete diet *ad libitum*. Attempts to determine with greater accuracy the nature of this interference should be pressed.

The Pennsylvania group (143), using Lansing virus, have shown that mice on a low protein diet (5% casein) exhibited a slight delay in onset of symptoms and those on low (0.4%) tryptophan exhibited a still longer incubation period, but eventually became paralyzed. The differences in their results as compared with ours may well be due to the use of a different virus and to the presence of an amount of tryptophan in the diet sufficient to provide a basis for the development of the typical disease. We have found that a diet containing as little as .05% tryptophan will not prevent paralysis in mice infected with Theiler's TO virus.

Other vitamin studies of significance include an observation of Pinkerton and Swank (146). Five per cent of 400 pigeons on a thiamine deficient diet for 5 to 12 days developed a disease similar to if not identical with psittacosis. Since the birds had seemed entirely well during a preliminary period of one to two weeks and had not been in contact with psittacosis, the authors suggested that the thiamine deficiency may have changed a latent infection with psittacosis virus into an active one.

Pinkerton and Moragues (91) have also reported a higher mortality rate and somewhat earlier deaths due to Lansing polio virus in normal mice compared with riboflavin deficient animals.

Saslaw (63) and associates observed that the absence of a nutritional factor, apparently folic acid, produced a marked leucopenia in *Macaca mulatta* rendering them susceptible to intranasal instillation of influenza virus. In normal monkeys, the virus produced only a transient leucopenia while in the nutritionally deficient monkeys with preinfection leucopenia, the virus produced marked lung involvement and death in 5 of 7 animals inoculated.

The evidence reviewed, coming as it does from many laboratories, is sufficient to make clear that nutritional deficiencies may definitely modify the usual course of several experimental virus infections. No over-all generalization can be made but the recent work brings to mind the earlier experiments of Zinsser indicating that unlike rickettsia and some other pathogens, viruses appear to thrive best under conditions of active tissue metabolism.

Manifestly, the conditions of many of the experiments are highly artificial so that one should guard against any suggestion that the facts obtained have any immediate application to the spontaneous disease involved. In most instances, however, the investigators have been studying diseases with severe infection and high mortality rates so that the challenge has been severe and less likely to detect minor differences in host-virus balance.

The interfering agents are so varied, some exceedingly simple, potassium and phosphorus, and others more complex, vitamins, vitamin analogues and metabolites in differing phases of nutrition, and some of the results so striking with almost 100% differences between controls and experimental animals, that one must urge more and continued study along many of the suggestive leads.

SUMMARY

A summary of reviews is inherently difficult. In this case, the great diversity of the experiments and the not infrequent inadequacy of controls, present such confusing and at times conflicting results that any attempt to generalize would seem premature. Mention should be made of two other recent reviews of this subject, those by Aycock and Lutman (149) and by Schneider (150). Further efforts to unravel the snarled skein should continue.

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IMMUNITY IN POLIOMYELITIS[1,2]

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Immunity in poliomyelitis is a particularly timely topic in view of recent studies from many laboratories. The theme of immunology presented here, however, will be launched from a series of events occurring on the small Pacific island of Guam, and subsequently subjected to laboratory and armchair analysis.

No attempt will be made to review much of the older literature on the immunity of poliomyelitis. As an excuse, a cue will be taken from Dr. Sabin, who said at the end of a Bela Schick Lecture (1), "I have drawn largely on my own studies, and may have left the impression that very little other work has been done on the subject that is worth mentioning". Then he quoted Professor Szent-Györgi who, when challenged at the end of a lecture by another scientist for not having mentioned his work, replied: "If I had taken time to describe his many contributions, I should have had no time left to tell of my own."

Articles about poliomyelitis are characterized by two common faults of popular medical subjects: excessive quantity and frequent lack of quality. The tremendous amount of trash cluttering up the literature renders all polio work most difficult. Rather often work of poor quality casts doubt on sound conclusions based on perfectly good work, but to remove the doubt it must be repeated, some times not just once but several times. The thousands of published articles nevertheless do include scores of reports of excellent work. In fact, the writings of Caverly, Flexner, Landsteiner, Wickman, Frost, Aycock, Trask, and many others provide precedent for practically every idea and every experiment reported since. Amateur scientists, however, many seeking easy publicity, have written profusely on poliomyelitis on the slightest scientific pretext.

Before launching into the subject of immunity in poliomyelitis, certain concepts should be clarified. First, infection with the virus of poliomyelitis must be clearly distinguished from the recognized paralytic or nonparalytic disease. Practically everyone eventually, and frequently very early in life, becomes infected with the several viruses, which we call poliomyelitis viruses, but the clinical syndrome called poliomyelitis is a rare manifestation, perhaps only a "complication" of these infections. Second, it is quite possible that the factor of resistance which prevents the majority of persons from manifesting central nervous system disease or "complication" during infection is *not immunity*, for in poliomyelitis this non-specific factor of resistance presumably is ordinarily present at the time

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of the first experience with the virus. True, specific immunity, must be the result of previous infection. Its functions are not well determined, for it may either prevent reinfection or modify future infection and thus render the likelihood of disease (already quite unlikely because of the natural resistance) still more unlikely. To the epidemiologist, the role of immunity in preventing reinfection and the carrier state is by far the more important possible function.

For several decades, statistical epidemiological studies have been made on observed or reported cases of poliomyelitis and such studies are multiplying at a great rate. However, conclusions drawn from these studies are based on a whole series of unproved suppositions—some logical, some rather dubious. It is presumed, for instance, that diagnosis has been accurate and that cases have been uniformly reported from all areas and at all times. Some concepts of immunity in poliomyelitis are derived from such studies, and others are based on still other assumptions. Some previously held concepts will be challenged and an attempt will be made to substitute or suggest alternate hypotheses for some of the present weak ones.

The first question I would like to raise is whether a single infection, clinical or subclinical, produces a lifelong or long-enduring immunity, such as that which occurs in measles. The second is closely related and can scarcely be separated from it: does poliomyelitis immunity prevent reinfection and the carrier state completely, again as we believe occurs in measles, or does it simply modify the clinical aspects of infection?

Because the age distribution of patients with clinical poliomyelitis had been observed to be roughly similar to that of patients with measles and chicken pox, it was postulated and generally accepted many years ago that immunity resulted from a single infection, apparent or inapparent, and that this immunity endured for life. Poliomyelitis has been listed in practically every textbook as one of the "immunizing infections of childhood". Acquired immunity appeared to satisfactorily explain the rareness of cases among older children and adults, an explanation accepted in the case of most acute communicable diseases of childhood. Proof of this is available, however, only in those diseases for which two characteristics may be demonstrated: (*a*) that adults are known to be just as susceptible as children if not previously exposed, otherwise immunity cannot be assumed to be responsible for a lower incidence among adults; (*b*) that persons having once had the disease must be observed to remain immune after many years' residence in an isolated environment where re-exposure and stimulation of immunity cannot occur, otherwise repeated, frequent exposure might account for apparent immunity. This proof is available in the case of measles, mumps and chicken pox, for there are reliable reports of outbreaks of these diseases in relatively isolated "virgin" populations, in which all age groups were equally involved. Several outbreaks of what has been *called* poliomyelitis have been reported in "virgin" populations (2–5). These also have affected both adults and children. Most of these were on tropical, Western Pacific islands. However, these reports must be considered with reservations because the infecting virus has not been isolated or identified, hence the disease could have been Japanese B encephalitis or one of

the many other infections of the central nervous system. We have recently demonstrated antibodies to Japanese B or other related viruses in sera from natives from some of these islands (6, 7). Most students of poliomyelitis today view these reports with considerable doubt about the validity of the etiological diagnosis (3). A more recent outbreak on the Nicobar Islands off the coast of India, referred to by Pandit, was the subject of laboratory investigation but the results are not yet known (8).

An opportunity has been presented recently in a series of visits to the island of Guam to observe or obtain information regarding an interesting series of outbreaks which stimulate thought on this problem. Mumps had not occurred in some parts of the island for 18 years, and any place on the island for possibly 8 years. American dependants probably introduced the virus some time in 1947. Practically every native not previously known to have had the disease, including adults, became infected, according to clinical or serological observations, the latter made in our laboratory. Those previously infected were immune. At the same time there occurred an outbreak of Japanese B encephalitis (6, 7, 9) which might have been reported eventually as poliomyelitis, as was an outbreak in 1899 (10), had there not been alert Navy doctors present. Unless consultants had been called, it might even then have gone on record as an epidemic of mumps meningoencephalitis. Next, measles came to Guam after an absence of 15 years, and similar observations to those of mumps were made. Children and adults alike developed the disease unless they had had it previously.

The next incident to occur on Guam, I consider highly significant. Poliomyelitis appeared about 6 months ago, but in this instance was manifest almost entirely among American children and adults, *not* among Guamanians as were measles and mumps. Finally, only four possible cases of poliomyelitis were found among the natives (11, 12). Yet, Captain C. K. Youngkin, Navy Medical Officer and Director of the Department of Public Health on Guam has told me that prior to this outbreak he has never been able to find any evidence of paralytic poliomyelitis among Guamanians although he has looked for it carefully. Before the outbreak we had tested serum from the native children for neutralizing antibodies to the Lansing strain of the poliomyelitis virus and found them present in children much younger than in the United States. Every serum of the small group then tested, beginning with those of 1 year of age, through 4 years, was positive. These same sera have been tested for antistreptolysin "O", and high titers of this antibody appear at a much earlier age than in sera collected in the United States. These laboratory studies will be discussed in more detail later.

Diphtheria infection, too, is present on Guam though seldom recognized clinically. Jacobziner (13), through the use of the Schick test, found that very few children even at 1 to 3 years of age are Schick positive (susceptible) yet the disease was not recognized and no artificial immunization had been practiced.

In contrast then to measles and mumps, poliomyelitis, diphtheria and beta hemolytic streptococcal infections, though attacking the population at an earlier age than in the United States, have *not* died out in this limited, semi-isolated population. Possibly the convalescent carrier stage of these infections accounts

for their persistence on Guam. This I doubt, however, for the carrier states are usually relatively brief, although a very few may remain carriers of diphtheria and streptococci for many months. If we presume that acquired immunity resulting from infection prevents reinfection and is permanent as in measles, these infections should certainly have to die out on Guam, until reintroduced.

Immunity to diphtheria has been studied intensively for many years, yet knowledge regarding the duration of naturally acquired active immunity and the effect of this immunity on the development of the carrier state is still poorly understood. It is generally accepted for diphtheria that many Schick negative (immune) persons *can* become reinfected and so serve as carriers. Both antitoxic and antibacterial immunity are probably involved; the latter very inadequately studied. The most apparent function of immunity in this infection is protection against clinical manifestations, principally due to the toxin. Most healthy carriers have antitoxic immunity. Duration of this naturally acquired immunity without stimulation by reinfection is not well defined, but there is at least one record of a large number of children who became Schick negative as a result of infection (not artificial immunization), reverting to the Schick positive state in 4 years (14).

Much less is known about immunity in streptococcal infections and the problem is greatly complicated by type specificity and the many types of demonstrable antibody, but, in general it is conceded that immunity may not prevent reinfection and the carrier state. In all probability, in diphtheria and in streptococcal infection, immunity is quite variable, tends to be temporary and is renewed by reinfection. It is also probable that certain levels of immunity, though adequate to modify disease, are not adequate to prevent infection. By inference, from the example of Guam, let us substitute for the hypothesis of permanent complete immunity for poliomyelitis, as in measles and chicken pox, one which holds instead that poliomyelitis immunity resembles that of diphtheria and streptococcal infections. Are there data which would invalidate or further support this hypothesis?

Age trends in disease may be affected by many factors, including age of susceptibility, age of exposure, and the duration of immunity. Therefore, one source of data in regard to duration of immunity may be the study of age trends. Let us consider briefly what comparisons with measles, diphtheria and streptococcal infection may contribute.

It has been pointed out repeatedly by Frost (15) and Aycock (16) that the modal age of poliomyelitis is generally higher in rural than in urban areas. Collins has pointed out that poliomyelitis occurs earlier in the children of low income families than those of the well to do (17). Doull (18) appears to have first pointed out that poliomyelitis together with diphtheria and scarlet fever occurs at an earlier age in the southern United States than further north, also at an earlier age in the tropics. Others since then have also pointed out this trend (19–22). We have been carrying out statistical studies recently in respect to the North and the South in the United States, examining race, population density and other variables. At their present stage these studies (23) confirm the earlier observations, suggesting that climate *per se* is one of the factors which influences the age at which poliomyelitis infection is acquired.

Numerous students of poliomyelitis from several different parts of the world have recently pointed out that a shift has occurred in the age distribution in their area since the earlier decades of the century. It is pointed out that a much higher proportion of cases now is reported in the age groups over 5 than formerly. This trend has also been apparent in California, on the basis of some of our studies (24). However, not all evidence supports this (25–27). Increased reporting of non-paralytic cases, which are more common above 5 years, and changes in the age composition of the population may accout for all of the changes. But, whatever the variation may have been in the age distribution of poliomyelitis, it is probably true that an earlier modal age indicates more rapid spread of the virus in the community, leading to earlier immunity. If immunity is complete and permanent the susceptibles are rapidly exhausted and the rates will fall rapidly in older children as in measles. If immunity is relatively temporary and the carrier state not prevented, it will be constantly renewed by reinfection, where spread occurs most readily, but may wane and lapse where a number of years occur between exposures. Such conditions of waning immunity in some can be postulated in areas where the greatest number of cases is reported as occurring in late childhood and among adults. Now let us examine which of the other diseases of childhood most closely follows the observed age pattern of poliomyelitis.

Measles has always shown a higher age in rural than in urban areas (16, 28). I can recall no studies made on the relation of economic status to age of measles.

Measles has *not* been shown to occur at an earlier age in the Southern States *nor* in the tropics (18, 23); in fact, with the decreasing density of population in most southern and tropical areas measles has shown the opposite age trend to that of poliomyelitis.

Some of our graduate students have been assigned the project of studying the age trend of measles in certain states since the early part of the century, to see if any change could be noted. Data have been difficult to obtain, but it appears that the only trend that can be noted is towards a greater concentration of cases at 5 or 6 years, the previous mode, probably due to better reporting in school age groups. There is thus no observable change to a younger or to an older age.

For diphtheria there is also good evidence for its earlier attack in urban than in rural areas (18, 28). The Schick test has also added to this evidence (29).

In contrast to measles, diphtheria shows the same trend as poliomyelitis in respect to latitude and climate (18, 30, 31). Despite the decreased density of population in southern states and in the tropics, the age at which observed infection occurs is lower, and the Schick test becomes negative at an earlier age (18, 30, 32). It was pointed out before that this early change of the Schick test had occurred on Guam (13). It should be noted as a further parallel that clinical diphtheria, like clinical poliomyelitis is observed less frequently in the true tropics, despite the more rapid dissemination of the infectious agent.

In respect to changes in the age distribution of diphtheria over a period of many years, little can be learned from American records, because artificial immunization has seriously disturbed the pattern.

Since streptococcal infections may also behave in a similar way to diphtheria and poliomyelitis and since artificial immunization has been practiced to such a

negligible extent, the parallels here may be studied more completely. Urban and rural comparisons of scarlet fever, by reported cases (28) and by Dick tests (33) closely parallel those of poliomyelitis and diphtheria.

Zingher (34) has pointed out as a result of Dick test surveys that there are more susceptibles among children of the well-to-do than among those of the lower income group, another parallel to poliomyelitis (17).

From many sources—Brazil (31, 32), Africa, (35, 36), China (37), the Philippines (30), and the United States (18, 38), it has been observed that in general, in the warmer or tropical climates, Dick tests become negative at an earlier age than in the more northern areas. Despite the fact, then, that clinical streptococcal infection is less common in areas of hot climate, we have indirect evidence that under these conditions there is in general, earlier and more rapid dissemination of streptococcal infection. This evidence is of course based on the validity of interpreting a negative Dick test as an immune response. Such an interpretation is accepted by most workers in this field, but is not accepted by all. Mortality and morbidity data also support this, since the percentage of deaths and cases reported due to scarlet fever under 5 to that over 5, is higher in those warmer areas as where such comparisons have been made (16).

Not having found any analysis of scarlet fever age trends in the northern United States, covering a period of many years, we have recently made a brief study of this.

Connecticut and Massachusetts are the only states where we have found ready access to annual scarlet fever morbidity reported by single years of age up to 10. In Connecticut (39) from 1924 to 1945 there has been no change in the modal age—always a peak at either 6 or 7 years of age. In Massachusetts (40) the modal age is 6 from 1932 through 1940. It was the same in Maryland from 1908 to 1917 (28). Thus, based on one crude method of examination no changing trend is noted. New Jersey (41) reports for cases by five-year age groups have been examined about the census years 1920, 1930 and 1940, employing reported cases for the census year, the year before, and the following year. Using age specific morbidity rates (table 1), and examining the ratio of the rate from 0–4 years to that of all ages above 4, the ratio was found to increase with time from 2.2 in 1920, to 2.5 to 3.5, indicating that reported cases are now more concentrated below 5 years than above. Mortality data (table 2) show no change between 1920 and 1940, the age ratios for the three census periods being 9.9, 9.6 and 10.0 respectively. Mortality records are certainly more accurate than morbidity, but they do not necessarily give the same information. California[3] statistics point to a lower age for reported scarlet fever in recent years. These morbidity statistics fail to conform with the generally held opinion of the pattern of poliomyelitis, but we must recall that conclusions drawn on the basis of such data are founded on a long series of assumptions of a very treacherous nature, and that certain careful investigators have pointed out hidden errors in some of the poliomyelitis data (25–27). It should be recalled also that scarlet fever may not be representative of all group A beta hemolytic streptococcal infections.

[3] Tabular data received through the kindness of Miss Geraldine Edwards of the California State Department of Public Health.

The difficulties encountered in interpreting morbidity and mortality statistics force us to seek evidence of a more direct and satisfactory nature. This it was felt might possibly exist in a serological test. At our request, Dr. Nell Hollinger of the School of Public Health has been kind enough to run tests for antistreptolysin "O" on sera which we collected for poliomyelitis antibody studies.

TABLE 1

Twenty year scarlet fever morbidity trend—New Jersey

	THREE YEAR PERIODS		
	1919–1921	1929–1931	1939–1941
Population*			
0–4 years	338,696	329,668	256,264
5+ years	2,817,204	3,711,666	3,903,901
Mean cases†			
0–4 years	1,277	1,350	1,482
5+ years	4,806	6,048	6,448
Rates per 1000†			
0–4 years	3.8	4.1	5.8
5+ years	1.7	1.6	1.6
Ratio of rates			
0–4:5+ years	2.2	2.5	3.5

* Population of census years, 1920, 1930, and 1940.

† Cases based on 3-year average.

TABLE 2

Twenty year scarlet fever mortality trend—New Jersey

	THREE YEAR PERIODS		
	1919–1921	1929–1931	1939–1941
Population*			
0–4 years	338,696	329,668	256,264
5+ years	2,817,204	3,711,666	3,903,901
Mean deaths†			
0–4 years	73	28.7	5.9
5+ years	61	33.6	9.1
Rates per 100,000			
0–4 years	21.5	8.71	0.23
5+ years	2.2	0.91	0.2
Ratio of rates			
0–4:5+ years	9.9	9.6	10.0

* Population of census years, 1920, 1930, and 1940.

† Deaths based on 3-year average.

The true significance of antistreptolysin is still unknown but in general, "the age of distribution of antistreptolysin is similar to that of naturally acquired diphtherial and scarlatinal antitoxin" (42). It is transmitted from mother to infant and gradually decreases in the infant (43, 44), as occurs also with antitoxin. However, antistreptolysin is not the same as antitoxin (45). The titers tend to

remain at a fairly constant level until a streptococcal infection occurs, then it rises much higher within 18 days and remains elevated for weeks or months (44). It has not been used extensively before for this type of survey work. The technic used in the test is that of Rantz (46). Results available at this time on the first 500 sera of our series to be tested have been analyzed for this preview, even though over a thousand sera remain to be examined. Equal numbers of serum specimens were collected from children at each single year of age, beginning at different age levels in different places, depending on results of earlier small samples tested for poliomyelitis antibody. In order to smooth out irregularities still present because of the relatively small numbers tested so far, the figures given are the result of applying the well known cumulative method of Reed and Muench for determining a 50 per cent endpoint (47). In this instance the age at which 50 per cent of the sera would have antibody has been roughly determined. Using 100 units or above as an arbitrary level of antibody, for division in two groups, the following results were obtained (table 3). In San Diego, California[4], a southern city, 50 per

TABLE 3

Age of development of antistreptolysin "O" in sera of normal children*

	AREA AND ECONOMIC LEVEL						
	San Diego		Bakersfield		Texas	Mexico City	Guam
	Upper	Lower	Upper	Lower	Mixed	Mixed	Mixed
Age in years by which 50% develop antibodies. .	6–7	4–5	6–7	3–4	5–6	3	2
Number of sera tested	68	81	109	39	90	76	36

* Antistreptolysin titer of 100 units or more.

cent of the children of the upper income families attained this titer between the ages of 6–7, while in the lower income families it was between 4–5 years. What this "economic" factor is we will not discuss just now, but you will recall that in studies by Collins (17), this was shown to correlate with poliomyelitis age distribution and the same had been revealed by the Dick test (34). In Bakersfield, California[5], over 200 miles further north but in the San Joaquin Valley where there are high summer temperatures, the upper income group paralleled San Diego, the 50 per cent age falling between 6 and 7, while the lower income bracket, representing here both whites and Mexican families in "shanty town", fell between 3 and 4 years, one year below San Diego where white Anglo-Americans only were included. A group from central Texas[6], mixed income bracket, fell

[4] Sera collected by members of the staff of the San Diego City and County Health Department, Dr. Alex S. Lesem, Director.

[5] Sera collected by Dr. W. C. Buss of the Kern County Health Department and by Dr. John Forney, with the assistance of Miss Mary Huston, R. N., Dr. C. I. Mead and Dr. R. L. Forney.

[6] Sera sent by Dr. J. V. Irons, Director of Laboratories, Texas State Health Department.

between 5 and 6 years, a mean of the high and low income groups for the other two southern areas. No series has been completed for the more northern San Francisco Bay area, but from evidence in Rantz's work, it is expected to be definitely higher. In Mexico City[7] a relatively high tropical city, the 50 per cent age is 3 years, and in Guam 2 years. We thus have preliminary evidence of an immunologic or serologic nature that streptococcal infection occurs at a very early age in the tropics, though the disease is seldom observed, and that infection spreads less rapidly in the more northern areas where much more disease is manifest.

The immunologic, clinical age, geographic and socio-economic parallels between poliomyelitis, diphtheria and streptococcal infections thus become even more closely established. We will examine those for poliomyelitis and streptococcal infections more carefully, later, in a single chart.

Up to this point I have attempted to avoid references to experimental laboratory, and serological evidences of immunity in poliomyelitis. Before turning to the laboratory, let us refresh our minds on the proposed hypothesis. Immunity is temporary; its maintenance depends on repeated infections, it may not prevent reinfection, though it probably does modify clinical response; the healthy carrier state is common among "immunes". This is put forth as an alternate to the older hypothesis of permanent immunity against disease and the carrier state, from a single infection. Based on statistical observations and "shoe leather" epidemiology alone, without reference to laboratory tests in so far as poliomyelitis is concerned, this proposed concept appears to explain findings at least as well as the older one. At this stage neither should be rejected completely in favor of the other.

Now let us see if the laboratory can offer some help in these problems of immunity and epidemiology in poliomyelitis. Soon after the monkey was found to be a susceptible experimental animal, neutralization tests were performed with human and monkey serum. Aycock and others employed this tool in epidemiological studies, usually using one monkey per serum sample. This tool was crude and the studies were limited by the cost of monkeys. Yet, conclusions drawn from this work have been supported and confirmed by the later and much more extensive studies using large numbers of mice in each test and the Lansing strain of virus. It was observed in the course of the early studies that the proportion of persons with antibodies increased with age and did so more rapidly in urban than in rural areas (48). Also, that if the maternal blood contained antibodies, serum of the newborn infant also contained antibodies (49). These disappeared by the time the infant reached six months of age. Although clinical disease occurred less frequently in southern states, infection occurred just as frequently (50). In other countries and on relatively isolated islands it was found that blood serum of adults regularly contained antibodies (51). Results of these surveys paralleled in a general way those of history surveys of infection in measles and

[7] Sera collected by Dr. Gerardo Varela, Director, Institute of Hygiene and Tropical Diseases, Mexico City, and arranged for by Dr. Wilbur G. Downs, International Health Division, Rockefeller Foundation.

as mentioned earlier, of negative Schick and Dick tests in diphtheria or streptococcal infection.

Those who performed these neutralization tests for poliomyelitis usually interpreted the results to indicate that specific immunity had resulted from infection. However, others, including very capable investigators, considered these poliomyelitis neutralizing "antibodies" to be entirely nonspecific or due merely to "maturation". These latter opinions grew to a large degree because a correlation between antibodies and clinical infection could not be observed. Convalescent and normal persons both had antibodies and usually of equal titer. Antibodies were found in the blood of people in areas where the clinical disease had *never* been observed. Still more significant appeared to be the fact that with few exceptions results of serologic tests at the onset of illness usually were just exactly like those at any time during or after convalescence. In most instances all serum samples from the infected patient either failed to neutralize or were positive. These observations differed greatly from those in other viral diseases associated with demonstrable serum antibodies. So, results, which seemed almost incompatible with specific response to infection, yet had much to suggest that they were the result of infection, were then interpreted by some to be due to differences in "strains" of virus—one group suggesting that the original "human" strain changed in the laboratory by monkey adaptation, another that fundamental immunologic differences, independent of the series of monkey passages, existed between the strain used in the laboratory and that infecting the patient. In other words this latter group considered that the test as usually performed was like using a laboratory strain of Western equine virus when dealing with cases of Eastern equine or St. Louis encephalitis. Some support for this interpretation was obtained from time to time and within recent years the evidence for it has become very convincing.

Since the very important contribution of Armstrong (52, 53), by using the mouse-adapted strains of virus, the earlier, more limited work has been widely extended and confirmed. Doubts are still expressed regarding some interpretations, but considering all the results, the specificity of the test and its usefulness as an epidemiologic tool have been generally accepted and, in my opinion, rightly so. For years, the use of the Lansing strain was challenged. It was called "atypical" and a strain from which conclusions about poliomyelitis could not be drawn safely. However, when the MEF-1 (54) the Philip (55), the W. W. (56), and the Wallingford (57) strains were isolated and the Yale S-K strain adapted to mice and typed (58), and when all were shown to be immunologically identical to the Lansing strain and all were cotton rat and mouse adaptable, many misgivings lost their reasonable basis. Most workers feel now that the Lansing type viruses do produce clinical poliomyelitis in man in many parts of the world, in addition to producing mild infection and antibody. The Lansing type virus has finally been granted a dignified and honorable position among other poliomyelitis viruses, even though stigmatized by rodent pathogenicity (59).

To trace the past of antibody studies in our laboratory at the Hooper Foundation, let us begin with a report of Lansing neutralization tests on acute and

convalescent phase serum from 23 patients in Washington and California in 1941 and 1942 (60). Prior to these dates, serologic tests on paired serum specimens had been reported in only single cases or smaller groups. Development of antibodies or rises in titer were detected in 10 of our 23 cases. Cautious, but over optimistic conclusions were drawn: "The practical application of the mouse neutralization test as a laboratory diagnostic method is obviously limited . . . . As to the application of this test to population surveys, it seems probable that it will serve in the same way as has the monkey test and possesses no advantages other than those resulting from economy." This report has remained unique and for reasons which will soon become obvious was probably considered by many to have involved some error in technic, but the results had been checked and double checked before they were published. Shortly thereafter, Turner and his co-workers (61) from Baltimore reported a much larger series composed of 64 pairs of acute and convalescent sera. Members of this group titrated the serum more completely and presented a detailed companion paper (62) emphasizing the reliability of the test as they performed it. These investigators did not find significant changes between the titers of acute and convalescent sera. The results subsequently reported by Brown and Francis (63) in 74 cases were similar to Turner's. Meanwhile, we made tests on paired sera in 102 additional cases and once again reported the results (64) and more recently Pait, Kessel and Grossman tested pairs of serum from 70 cases (65). This time our results, and those of Pait, Kessel and Grossman, agreed with those of Turner, Brown and Francis. To us it appeared obvious that, except for part of our original series, the results reflected an immunologic difference between the Lansing strain used in the test and the strain of virus that had invoked the illness. However, in our report we expressed certain misgivings regarding the significance of some of the antiviral activity of certain human and animal sera. By then more information was available to support the multiple strain hypothesis, notably that from Kessel's group (66) and an earlier very complete and convincing review by Aycock (67).

At the same time that our last series of tests of patients' serum was carried out, we reported antibody tests on a relatively large series of monkeys infected in the laboratory with various strains (64). Only those infected with Lansing, the MEF-1 and one other virus from Nebraska (human cord material which could not be adapted to monkeys) produced antibodies protective against the Lansing strain. These results added evidence for the strain specificity of the test in monkeys at least.

Even before this, we were convinced that, if during infections with most types of poliomyelitis virus, antibody changes occurred in man, they would have to be demonstrated by using virus of the same immunologic type as that responsible for the infection. We had, therefore, begun a long term project aimed at clarifying this fundamental and crucial problem. Preliminary results of this, after 3 years of work, have been reported recently (68).

Stools and acute and convalescent phase serum were collected in over 50 cases of paralytic poliomyelitis. Virus was isolated from many of the stools, and as many strains as possible were sufficiently adapted to the monkey by serial passage

to permit performance of a satisfactory neutralization test with monkeys. Antibodies against the homologous strain increased in all but one instance in the small series which was finally completed. Unfortunately, this project was beset by many unforeseen obstacles. Most of the twenty-some strains isolated in the three outbreaks studied never became sufficiently pathogenic for the monkey to be suitable for neutralization tests. Two monkey "famines" occurred and most of the monkeys obtainable on the West Coast, it appears in retrospect, were those

TABLE 4

Monkey neutralization tests with acute and convalescent phase sera from patients, tested against homologous viruses isolated from the same patients

PATIENT AND VIRUS	RESULTS* MONKEY SERUM CONTROLS	PATIENT'S SERA					
		Acute			Convalescent		
		Days after onset	Serum dilution	Results*	Days after onset	Serum dilution	Results*
4	4/4	5	Undil.	0/2	74	Undil.	0/2
			1:5	0/2		1:5	0/2
			1:25	2/2		1:25	0/2
			1:125	—		1:125	2/2
			1:625	—		1:625	2/2
5	3/3	4	1:5	1/2	24	1:5	0/4
			1:25	2/3		1:25	3/4
6	4/4	4	1:3	0/3	80	1:3	0/3
			1:15	3/3		1:15	0/3
			1:75	1/2		1:75	0/3
7	4/4	4	1:3	0/3	45	1:3	0/3
			1:15	2/3		1:15	0/3
			1:75	3/3		1:75	0/3

* Numerator represents number of monkeys developing paralysis and the denominator the number inoculated.

Table adapted from: Hammon, W. McD. and Roberts, E. C.: Serum neutralizing antibodies to the infecting strain of virus in poliomyelitis. Proc. Soc. Exp. Biol. Med., 69: 256–258, 1948.

which laboratory workers nearer the source had discarded as unfit for use: they were still less fit when they arrived in San Francisco.

Sera from the first three patients were tested as we ordinarily test for antibodies when working with the encephalitis viruses, the standard method used to that time with poliomyelitis virus. The undiluted serum was mixed with 20 per cent monkey cord suspension and sometimes with an ultracentrifuged concentrate of this when the pathogenicity of the strain was low. In such tests serum obtained during both the acute and convalescent phases completely neutralized the virus. We were surprised by these results, so we repeated the tests until there was no more serum. The results were confirmed. All suggestions in the literature were to the

effect that antibody formed slowly in the monkey, and probably in man. Why then, if we had the right immunologic type of virus, did the patient have antibody at the onset of illness?

In the next four cases (table 4) serial 5-fold dilutions of serum were tested. Here it was again observed that each one of the samples obtained during the acute phase contained antibodies, but in 3 of the 4 instances, antibody was present in lower titer than in serum drawn later.

Finally, by this experiment the fact appeared to be established that in man neutralizing antibodies do increase as a result of infection. *We*, at least, were not convinced in respect to this point until completing these tests. The reason all our patients exhibited antibodies so early in the course of the disease still remains to be found. But, in this respect, poliomyelitis is parallel to Western equine and, to a lesser degree, to Japanese B encephalitis as these infections occur in horses and man. In these two other neurotropic virus infections antibody can usually be detected in significant titer in the first serum specimen.

Having heard that Steigman and Sabin had recently made tests on human serum by a method somewhat similar to one used in our series, I asked for an opportunity to see their results and Dr. Sabin was kind enough to send an abstract of a paper which had just been read (69). Out of 9 cases studied, antibodies were demonstrable in the first serum in all but two cases, but a rise in titer was found in all during the acute phase of disease, thus confirming our findings. They also found that many of the strains of virus isolated were unsuited for tests of this type in monkeys, since the paralytic rates which they produced were exceedingly low.

All of this work lends great assistance to the interpretation of the many incomprehensible results previously reported on the development or lack of development of neutralizing antibodies in human serum during the course of infection. It now appears clear that antibodies are at least partially strain-specific or type-specific, usually develop early in the disease or are already present in low or moderate titers at the time of exposure, owing to a previous infection.

Tests made with laboratory strains from other cases as routinely performed in the past cannot be expected to yield comprehensible results. Neither can it be assumed that a virus isolated from another patient in the same outbreak will be satisfactory (70), for several strains may be and probably are active in many epidemics.

Results of these recent monkey neutralization tests help to clarify the reason why a number of capable investigators were so puzzled by their own results and were led to believe that neutralizing antibodies for poliomyelitis viruses were entirely nonspecific and meaningless.

Now that we can safely assume that antibodies to the Lansing virus result from a poliomyelitis infection let us see what antibody tests can contribute to comparing the trends of poliomyelitis infection with those of scarlet fever and diphtheria.

So far the population of no region in the world has been found free from

poliomyelitis antibody, although in some of these areas clinical poliomyelitis has not been observed or has been only rarely observed. Nevertheless in a number of such areas Americans or Europeans have developed the disease with a notable escape of the intimately associated natives. This strongly points to the likelihood of immunity on the part of natives. Gear (71) has reported rarely finding cases of poliomyelitis among natives of tropical Africa, although high rates might occur in whites. In one area completely free from cases, he demonstrated antibodies in the natives, then virus in the feces of a few, proving that infection is active among them even though completely inapparent. Three out of nine children tested by Gear were excreting virus when tests were made! This closely parallels the tropical situation for diphtheria and streptococcal infection.

Sabin (72) has mentioned that he and Young have tested about 300 sera from children of the Far East for antibodies to the Lansing strain and found that antibodies developed earlier there than in Cincinnati and earlier than in a normal group of Baltimore negroes tested by Turner and associates. We collected sera from some of these same Far Eastern areas, also from Guam, Kwajalein, Amer-

TABLE 5

Age of development of Lansing neutralizing antibodies in sera of normal children*

	AREA AND ECONOMIC LEVEL							
	San Diego		Bakersfield		San Francisco	Texas	Mexico City	Guam
	Upper	Lower	Upper	Lower	Lower	Mixed	Mixed	Mixed
Age in years by which 50% develop antibodies*.	6–7	5–6	7–8	4–5	10–11	7	2–3	<1
Number of sera tested.	76	61	92	92	65	84	81	84

* Lansing Neutralizing Antibodies: Neutralization 10 to 100 LD_{50} or more.

ican Samoa, Mexico and several cities in the United States to attempt to test the correlation of age of developing neutralizing antibody with the age of reported infections. Tests on over a thousand of these sera are still incomplete but a prevue of 635 will be offered here (table 5). It appears from these partial results that antibodies develop much earlier in the tropical Pacific Island of Guam (over 50 per cent positive at 1 year of age) than in northern United States particularly San Francisco (50 per cent age in the low income group between 10 and 11), and it also appears that in California antibodies develop earlier as one proceeds south since in Bakersfield and San Diego the upper income group has a 50 per cent age for antibody between 6 and 8 and the lower income groups between 4 and 6. Where reporting of cases occurs this age distribution correlates very closely with the age of the recognized and reported cases. We reviewed these ages recently for 4 California cities (24).

In the tropical Pacific Islands where the disease appears to be *very* rare, the antibodies appear *very* early. I believe we may assume that infection in these areas occurs at an extremely early age, and disease, if it occurs at all, is confused

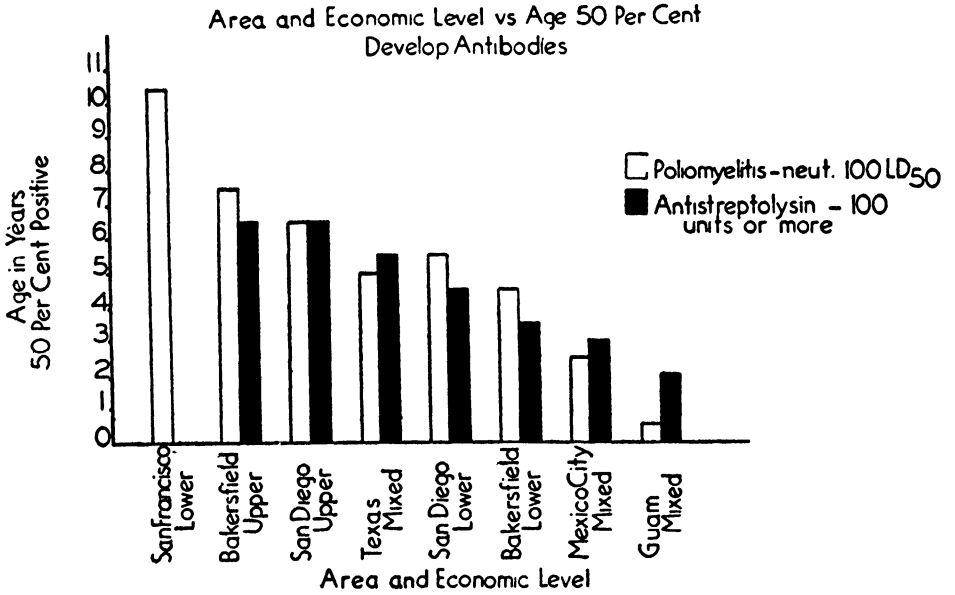
with many other ailments of infancy. Poliomyelitis may even be responsible for a certain amount of the high infant mortality rate. In any case, the association of age of infection where known, and the age of developing antibodies to the Lansing virus appears to be excellent.

Next, let us note the correlation between the age of infection and the economic status of the family. The antibody tests in San Diego and Bakersfield support well the family survey findings reported by Collins (17). Infection develops earlier in poor families. Family size is quite possibly a contributing factor of the economic complex.

Now, let us examine further the correlation of this evidence of an immune response with that of antistreptolysin. Chart 1 presents the results of tests in

CHART I

LANSING NEUTRALIZING ANTIBODIES AND ANTISTREPTOLYSIN "O" IN SERA OF NORMAL CHILDREN



each area and each economic group with antigens of the two infectious agents. The correlation by age, by place and by economic status is most remarkable. If the results continue to bear this relationship when these and additional series on hand have been extended as planned, it would appear highly probable that so many correlations indicate similar epidemiologic patterns; including the effect of immunity on the carrier state and on its duration. It is not intended to infer that the streptococcus and poliomyelitis are etiologically related, in fact this is entirely contrary to our interpretation of the data as will be pointed out in detail in subsequent analysis.

Now let us consider what role neutralizing antibody plays in the immunity of man. I have the temerity to refer again to the arthropod-borne encephalitis

viruses, with which we have had more experience and have developed most of our technics. This is done despite sharp verbal criticisms received in the past from some workers to the effect that the poliomyelitis virus is so entirely different that to suggest parallels with another neurotropic virus is misleading and confusing. This criticism, for the most part, preceded the enlightenment regarding the real significance of neutralizing antibodies and was made at a time when type differences, which have long been recognized in the encephalitides, were not so well established for poliomyelitis. At the moment, it appears quite significant that every one of the immunologic technics, tests, and devices used in encephalitis studies is being employed to advantage in poliomyelitis, including immunization with formalinized or live vaccines, then cross challenge, quantitative serological test of several types, even including complement fixation, and in addition, flocculation (a recent development by Dr. E. C. Roberts in our laboratory). These developments have come in a large part through the work or ideas of several investigators working simultaneously or previously with other types of neurotropic viruses.

For the arthropod-borne viral encephalitides there is considerable reason to believe that neutralizing antibodies play an important role in the immunity mechanism. Here the virus is introduced into the blood or tissue spaces, where it may be inactivated by serum antibodies, probably before it comes in contact with susceptible cells. Furthermore, passive serum immunity to experimentally produced infection by an exact reproduction of the natural route has been demonstrated.

In poliomyelitis, evidence of the role of antibody in immunity is as yet incomplete. It is difficult to be certain that the normal or "natural" route of entry or penetration of poliomyelitis virus has been determined; hence it cannot yet be stated with assurance that infection by the "normal" route has ever been reproduced in laboratory animals. Furthermore, it cannot be assumed with certainty that any laboratory animal is regularly most susceptible to infection through the same portal as man. The erroneous conclusions drawn after dropping virus in the nose of monkeys should remain fresh in our minds. Without knowing the route of invasion we cannot reason logically regarding the barrier that antibody may present to virus. Some, now believe that the infection invoked by feeding virus to chimpanzees serves as a true replica of normal human infection. However, these chimpanzee feeding experiments by Hopkins and Yale workers (73, 74) and similar studies with baby monkeys (75) are the best available at the moment and may serve as the basis for some conclusions about the immunologic mechanism, and, for cautious reasoning, with regard to what may happen in man. After the feeding of any of several types of poliomyelitis virus to chimpanzees (74, 76) and of one type to immature monkeys (77), type-specific neutralizing antibodies developed promptly. After a reasonable interval the virus was shed in the feces for days or weeks, even though the animal showed no signs of illness. In the baby monkey, at least in those in which paralysis accompanied the infection, antibodies were present by the time paralysis occurred (77), although it customarily develops much more slowly in animals inoculated intra-

cerebrally. This same early antibody formation, it should be recalled, was revealed in our tests on normal human paralytic infections (68). The chimpanzees and monkeys, although now endowed with antibody from infection with the original virus strain, when fed a different immunologic type of virus again became carriers. However—and this would appear to be the significant feature—these animals, when fed the same type of virus, within a few months, did not again pass virus in the feces, with only one exception (76), suggesting that they were *immune to reinfection*. At the moment, there seems to be no way to determine whether antibody played any part in preventing infection or interfering with virus fed to these animals in their food. However, in man the antibody found in nasopharyngeal secretions might play such a role.

Amos and Taylor (78) in 1917, and Howitt in 1937 (79) presented evidence for neutralization of poliomyelitis virus by human nasal, mouth and pharyngeal washings, and other studies have also been made on this problem. This neutralizing substance has recently been restudied by Bell (80) and finally identified by him as specific antibody. One may readily speculate on the effect of this antibody found in man, which is so strategically placed in the mouth, throat, and nose, bathing the mucous membranes where the virus may have first contact or may actually penetrate or find receptive cells.

One may also speculate as pointed out by Bell (80) regarding the effect upon the virus which is being discharged from these mucous surfaces during the early stage of infection as such antibody develops during the course of primary infection. Development of antibody may explain the early disappearance of virus from the throat, and thus control this source of infectious material. The fact that virus persists in the throat for only a very short time is well established.

Is there such a mechanism in the intestinal tract at a lower level, or do these antibodies from the pharnyx pass on into the intestines? This has interested us the past several years, under stimulation of the extensive literature on copro-antibody for cholera and bacillary dysentery. The poliomyelitis literature is remarkably silent on this subject. Details of our experiments of the past several years cannot be presented here but a few facts appear to have been established. Extracts of feces of normal monkeys have failed to neutralize Lansing virus, consistently. After establishing this, the MEF-1 strain of virus which is identical immunologically to Lansing virus, was used to inoculate a series of monkeys intracerebrally. This passage material had not been mouse adapted. Extracts of monkey feces collected at 8 and 15 days after paralysis have shown in repeated tests a protective effect of 1.5 to 2 logs against Lansing virus. It appears therefore that some substance with antiviral activity does appear in feces of monkeys inoculated intracerebrally with MEF-1 strain of virus. It remains to demonstrate whether this is, or is not, antibody. It is entirely too early to draw any conclusions about man from these results but it suggests that the feces of man deserve examination.

Morgan has furnished us with some of the best experimental evidence for correlation of immunity and antibody. By giving monkeys a series of intramuscular inoculations of live virus, she has shown a very convincing correlation

between the level of serum antibodies and the resistance to intracerebral inoculation (81), just as she had shown a similar correlation with one of the encephalitis viruses (82). It may be recalled however, that in some of the clinical cases in which we studied the antibody rise by monkey neutralization test with homologous virus, antibodies were readily demonstrable before paralysis, yet the disease progressed to the paralytic state (68). This may simply mean that at that time the antibody titer was not high enough to protect. However, as an indication that other mechanisms may be involved let us note that back in 1936 Sabin and Olitsky showed that convalescent monkeys were resistant to reinfection by the intranasal route before *any* serum antibodies could be demonstrated (83). They also pointed out certain other facts which did not correlate antibody with immunity to experimental disease. Further study of this problem is necessary but at present the possibility of cellular immunity playing a partial role must be strongly considered.

Morgan has recently demonstrated what she believes to be local antibody found in the tissues at the site of poliomyelitis lesions (84). In the intracerebrally inoculated animals these substances appeared before serum antibodies. However, in the light of the much earlier serum antibody formation in monkeys given virus by a more "natural" route (by mouth), by von Magnus and Melnick (77), the significance of Morgan's findings in respect to human infection is very difficult to evaluate.

It now becomes appropriate to consider more carefully the laboratory evidence for the duration, in man, of immunity to reinfection. Reports of several excellent studies (85–89) to detect virus in throat swabs, in feces, or both, made in families where a single case of poliomyelitis is recognized give us extremely interesting and valuable information. Tests for virus on a single specimen of feces or throat secretions collected at some time soon after recognition of the first case in the family, reveal that approximately 80 per cent of the children and 50 per cent of the adults are infected at the time of the single test. If specimens were taken repeatedly and more monkeys employed in the tests there is excellent evidence to indicate that higher proportions would be found infected. Such adult infection rates are extremely rare in measles. On the basis of tests for poliomyelitis antibody with any strain commonly employed, we would expect possibly 50 per cent of the children and 2 to 5 per cent of the adults to be free from antibody. This proportion correlates better with the relative expectation for paralytic poliomyelitis between children and adults, than it does with the observed infection rates (80% and 50%, respectively). So, at first glance, at least, it appears that a high proportion of adults, though possessed with neutralizing antibody and who are presumably "immune", are still susceptible to infection and develop the carrier state. This, to me is the strongest evidence we have of the lack of immunity to infection by those who by every indirect criterion can be assumed to have been previously infected. However, it will be recalled that the chimpanzees and monkeys became immune to reinfection after feeding, for they failed to develop the carrier state when fed the same virus twice. This would appear at first to conflict with the "human" evidence of immunity not preventing reinfection.

But, the laboratory animals were challenged after only a *short* period of time, not after several years as may frequently elapse between re-exposure to the same strain in man. Immunity may be at such a high level for a few months that it ordinarily prevents reinfection, but in its waning phase reinfection is not prevented. I expect this will be proven to be so. At this time it appears probable, in view of known facts, that immunity to reinfection is temporary.

Now, let us consider the duration of acquired neutralizing antibodies, a substance more readily measured. Unfortunately, we can find little evidence to bear on this problem. Man is subject to repeated exposure so continued high titers observed by a few of us in certain individuals mean nothing. I know of no record of repeated tests over a period of years on an isolated, convalescent laboratory monkey. Therefore, at the present, studies on neutralizing antibody do not help us to settle the question of the duration of even this evidence of naturally acquired immunity.

The only other way I can think of to determine duration of neutralizing antibody is to find an isolated island, if such exists, where no neutralizing antibodies to some *one* type of virus are present in the population, and keep there for a number of years a few uninfected individuals who have antibody, or find such an experiment already made to order. Such a situation, where even one immunological type of poliomyelitis virus was absent might answer many questions. Furthermore, the accidental introduction of the missing virus could answer still more questions if competent observers and collection facilities were available. The question regarding adult susceptibility would also undoubtedly be answered. I do not believe that an island lacking infection with one of the several immunologic types of poliomyelitis virus will be impossible to find, once we have a suitable inexpensive serologic test to use for the survey work. Such a test, I am pleased to say may be "just around the corner" on the basis of results which Dr. Roberts of our laboratory is obtaining with a type specific poliomyelitis flocculating antigen (90).

At this point a diversion will be made by way of illustrating an immunity mechanism occurring in another common virus disease. With Dr. John Enders for two years, and later alone, experiments were carried out on a common, world-wide virus disease of cats. Independently and concurrently with Lawrence and Syverton (91), we found this infection had as its chief pathology, destructive lesions of various elements of the hematopoietic system (92, 93). We called the disease panleucopenia. Lawrence and Syverton called it agranulocytosis, and undoubtedly it is the same disease named by others, infectious enteritis, though entirely inaccurately described by that name since there exists no inflammatory lesions. In our immunological studies we found we could passively immunize susceptible kittens by giving convalescent or adult "alley-cat" serum. Vaccination with formalinized cat spleen could also be carried out effectively if the animals were vaccinated several weeks before bringing them to the laboratory, but all susceptible rural cats died after laboratory exposure, unless previously immunized either by serum or by a previous series of vaccine injections (94). It was equally true that city cats and kittens even though the kittens were born in the

laboratory, apparently never became ill. The hypothesis was formed early that the new-born kittens in the city, where infection was apparently omnipresent, were endowed with maternally transferred passive immunity and that they developed a more permanent active immunity through mild or inapparent infection acquired in very early life while still possessing maternal protection (95). This was put to test by routinely inoculating rural susceptible kittens with immune serum once, when introduced into the laboratory. Large numbers of these animals were kept from 6 months to 1½ years and none ever became recognizably ill with panleucopenia (23). The speaker has therefore, long suspected that a similar mechanism might play an important role in immunity to poliomyelitis where conditions were most ideal for its rapid and easy transfer to infants. Some evidence has now been obtained to support this, on Guam at least. Here, all sera tested to date from children between 1 and 4 years of age contained neutralizing antibodies. Dr. Sabin reported that he was unable to demonstrate significant amounts of antibody between 9 months and 23 months in his sera from Japan, Okinawa and Korea and suggested that immunity was not acquired there at such an early age, but further south on Guam the conditions may be somewhat different. This means of safely acquiring immunity, therefore, does not appear to play any important role, except under relatively extreme conditions, probably restricted to certain tropical areas.

No attempt will be made to review in any detail the already extensive literature on the typing of poliomyelitis viruses that has appeared before and since the Virus Typing Committee of the National Foundation for Infantile Paralysis began holding meetings to plan standardized methods and experiments (57, 90, 96–100). By a combination of vaccination and challenge, challenge of convalescents and cross neutralization tests, it appears that at least three distinct immunologic groups of virus exist among the limited number of domestic strains so far tested. Within at least one of these groups there appear to be certain strain differences, revealed most readily by the neutralization and flocculation tests. Already it appears that infection with one can hardly be expected to furnish effective protection against another, any more than St. Louis encephalitis infection can protect against the Western equine type. However, group relationships like St. Louis, Japanese B and West Nile, with some slight antigenic overlapping will probably be found.

The big question in the mind of many is the practical application of all this knowledge to artificial immunity. The speaker's personal opinions and his reasons for them can be presented in a very few sentences.

We will, or we should hesitate a very long time before we use living virus again for poliomyelitis immunization. Inactive virus will produce antibody and temporary immunity to disease in monkeys if given in repeated and very large doses (101). However, a certain danger exists from a type of disseminated encephalomyelitis believed to be due to sensitization with brain tissue components which accompany the virus. Adjuvants added to the monkey cord suspensions have already induced highly severe and fatal lesions in monkeys after just one inoculation (102, 103). Next, killed virus vaccines of any disease result in a very

temporary type of immunity. This could mean that children would require annual vaccination, and once started might have to have it continued for life. To date, only members of the Lansing group of viruses (one immunologic type) can be produced in any animal other than the monkey, and no true poliomyelitis virus has been grown in the chick embryo. Thus, at least two types and possibly many more would have to be made from monkeys, in addition to the Lansing type which could be made from mice. For this the sources of monkeys in the world would be completely inadequate. Furthermore, only one child in hundreds, possibly thousands needs vaccine for protection against crippling, and we have no way to determine which one does. Thus, from the standpoint of danger, necessity of repeated administration, multiplicity of immunologic types, exorbitant cost, insurmountable logistics and difficulty in selection of susceptibles who need vaccination, it all appears to add up to an impractical and seemingly impossible undertaking. Some new development may, of course, completely alter the outlook, but in the meantime I imagine most of our children will be forced to get along with a normal naturally endowed high resistance, supplemented from time to time with a bit of naturally acquired immunity of variable duration and effectiveness. We adults will depend on the same mechanism and all of us may continue to spread virus periodically, at unpredictable times and under unexpected circumstances. On this type of hazardous experience the level of our immunologic status possibly depends, as for streptococcal infections, and until recently, for diphtheria.

It is the speaker's belief that the most practical immediate application of knowledge to be gained in the field of immunity will be in the development of better laboratory procedures—such as serologic tests, and immunologic means of detecting and typing viruses in diagnostic and epidemiological studies.

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THE "DELFT SCHOOL" AND THE RISE OF GENERAL MICROBIOLOGY[1]

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Antonie van Leeuwenhoek certainly started something when he began making his lenses and examining anything he could lay his hands on! His observations led, about 300 years ago, to the discovery of the "little animals", now known under the names of protozoa and bacteria. And thus Leeuwenhoek, the Delft draper and scientist, became the "Father of protozoology and bacteriology," as Dobell (1) has so aptly called him. There is no doubt that the science of general microbiology began in Delft.

It was an exciting beginning. The animalcules were found almost everywhere, and they appeared to represent an astonishing array of sizes and shapes. Practically any kind of material revealed their presence—a wonder to behold, a pleasure to watch. And Leeuwenhoek made the most of his discovery: witness the enormous number of letters which he sent to the Royal Society of London vividly describing his observations with many pertinent reflections upon their significance.

Yet we do not ordinarily think of Leeuwenhoek as the founder of a "Delft School", or, for that matter, of any school. He was a solitary worker, and occasionally even reluctant to disclose to others the methods he employed. In consequence we are, even today, confined to speculation when it comes to deciding whether or not Leeuwenhoek might have hit upon a way of examining specimens by using some sort of dark-field illumination, a possibility that was suggested by Dobell (1, p. 331–2). This suggestion has been more fully discussed by Cohen (2) and Kingma Boltjes (3).

Now, Leeuwenhoek did not start a "school," and so his methodology was handed down to posterity only insofar as his letters divulged. There were no pupils who might afterwards have revealed secrets which the master had decided not to publish. But his discoveries were so spectacular and so unexpected that they could not fail to fire the imagination of others, equally imbued with curiosity, that driving force of scientific endeavor. Hence his observations were, in the course of time, repeated and extended in other places, and knowledge concerning the microbes gradually accumulated, until today there is available an amount of information so vast that it would be impossible for a single individual to be conversant with more than a small part of it.

This is an unfortunate although inevitable result of expanding scientific activity: the interested individual must needs make a choice as to what shall occupy his mind and hands. Thus there is a real danger that he may become involved in minutiae; a narrow specialist who "knows more and more about less

[1] Based on the A. J. Kluyver Lecture delivered before the Society of American Bacteriologists. Cincinnati, Ohio, May 19, 1949.

and less." However, an increase in factual information, the only solid basis for scientific accomplishment, also brings with it the desire for organizing and integrating the details. If this is satisfactorily accomplished the isolated data can be connected together into a framework composed of general principles. And the latter mark the culminating advances of science.

In what follows I shall try to sketch the gradual development of some principles in the realm of general microbiology; to show how these are associated with a "Delft School"; and to indicate how they have contributed to the rapidly growing interest in this field.

Obviously, Leeuwenhoek's discovery of the existence of the "little animals" raised problems concerning their origin, their activities, and the significance of the latter. Leeuwenhoek himself expressed opinions on these questions that were essentially identical with those which, two centuries later, became the established scientific views. During the intervening years the issues were, however, ardently debated on the basis of seemingly conflicting experimental results, and these experiments have added greatly to our store of knowledge.

I shall not dwell upon the fascinating controversy about the spontaneous generation of the microbes *versus* their origin from preexisting ones. The battle, earlier fought over the origin of larger organisms, and quite recently again over that of viruses, ultimately led to an acknowledged victory of the proponents of the idea of biogenesis. In the meantime the discrepancies in the outcome of many crucial experiments gradually led to the development of an adequate methodology for the study of microörganisms. Most of the techniques now so confidently used represent modifications and refinements of methods that had once produced results interpreted in favor of spontaneous generation. Thus can the mistakes made in scientific investigations be turned to advantage, for they lead to the recognition of unexpected and unpredictable sources of error, and so permit the eventual elimination of the latter.

But the apparent defeat of the doctrine of spontaneous generation left unsolved the fundamental problem of the origin of life. In recent years new ideas have been expressed, notably by Haldane (4), Oparin (5), and Horowitz (6) which have a strong scientific appeal because they suggest a way out of what otherwise would be an impasse. Whether these concepts can soon be made the basis for a renewed experimental attack cannot now be decided; the answer must be left to future studies.

So much for the first problem. The question of the activities of the "little animals," too, was contemplated by Leeuwenhoek, and once again he reached a conclusion that was not to become part of our scientific outlook until two centuries later. I do not here refer to the concept that microörganisms can play a role as causative agents of disease, but to the far broader one concerning their function in the cycle of matter. It should be realized that the former activity represents no more than a very minor aspect of this general phenomenon.

The important part played by microörganisms in transforming organic and inorganic substances on earth with the result that these may be used over and over again to sustain life of other organisms was first clearly expressed by Fer-

dinand Cohn (7) in 1872. In thus making possible the continuation of the great experiment of evolution the "little animals" occasionally perform their task in a manner that clashes with the desires of man who, through ignorance and greed, has a propensity for eliminating various natural resources from participation in the natural cycle of matter, and often appears to regard the earth with all that is on it as his own private property. This has led to an unwarranted emphasis on such resented activities of the microbes as would interfere with man's hoarding instinct, even to the point of making him lose sight of the fundamental significance of an uninterrupted continuation of the cycle. Those who have learned to view life in a wider sense can but hope that, through education, a better comprehension may gradually be reached, and the hoarding instinct be curbed—if there is still time.

Our knowledge of the diverse types of microbes responsible for the specific major transformations of matter has advanced greatly since Cohn's pronouncement. The most important contributions to this problem we owe to M. W. Beijerinck (8), the second of the great Delft microbiologists. By introducing the principle of enrichment cultures he opened the way for a rational approach to microbial ecology. Although some of Beijerinck's specific discoveries are fairly well known to most microbiologists, the fundamental ideas that led to them have been appreciated far too little. This, I believe, is due to the fact that Beijerinck, who could have written a treatise on enrichment cultures that would not have failed to exert a profound influence, never so much as published a paper in which the principle was clearly formulated and its potentialities developed. When, in 1905, Beijerinck was awarded the Leeuwenhoek medal by the Koninklijke Akademie van Wetenschappen in Amsterdam, F.A.F.C. Went noted the above mentioned deficiency in his presentation address with the following words:

"There is in your publications such a wealth of original concepts and of special approaches, often buried in a couple of sentences, that such a treatise would surely be anticipated with the utmost interest. It would then also become clear how many of the current ideas in microbiology we really owe to you; this is far more than is apparent to those who merely have taken superficial notice of your publications" (9).

It was only on this occasion that Beijerinck stated his objectives and approach. I translate from his acceptance speech:

"I am happy to note that the way in which I approach microbiology has the approval of the best judges. This approach can be concisely stated as the study of microbial ecology, *i.e.*, of the relation between environmental conditions and the special forms of life corresponding to them. It is my conviction that, in our present state of understanding, this is the most necessary and fruitful direction to guide us in organizing our knowledge of that part of nature which deals with the lowest limits of the organic world, and which constantly keeps before our mind the profound problem of the origin of life itself. Therefore it is a great satisfaction to me that the Academy apparently wishes to honor the experimenter who exploits this field.

"In an experimental sense the ecological approach to microbiology consists of

two complementary phases which give rise to an endless number of experiments. On the one hand it leads to investigating the conditions for the development of organisms that have for some reason or other, perhaps fortuitously, come to our attention; on the other hand to the discovery of living organisms that appear under predetermined conditions, either because they alone can develop, or because they are the more fit and win out over their competitors. Especially this latter method, in reality nothing but the broadest application of the elective culture method, is fruitful and truly scientific, and it is no exaggeration to claim that the rapid and surprising advances in general microbiology are due to this methodology. Nevertheless, and this in spite of the fact that Leeuwenhoek, more than two hundred years ago, already used this aspect of micro-ecology in some of his studies, and that Pasteur was enabled to make most of his great discoveries because he was guided by the same principle, the number of conscious exponents has so far remained very small. And I feel that I certainly may be reckoned among them because of the enthusiasm that is in me to contribute to the grand task that can here be accomplished" (10).

That is all. And who would bother to read these sentences, representing half a page of a printed speech, and written, like Leeuwenhoek's letters, in the Dutch language, some 45 years ago? Beijerinck never got around to writing the treatise Went had suggested, probably because he was more interested in doing experiments, and so the number of workers who consciously applied Beijerinck's principles remained small, limited, in fact, to those who had the good fortune of experiencing his influence, directly or indirectly. It is true that in 1907 Stockhausen (11) published a number of essays on microbial ecology ("Enrichment cultures after Beijerinck") in the "Wochenschrift für Brauerei," also issued in book form. But thirty years after its publication the first edition of the book was still far from exhausted, and many microbiologists have probably never heard of it.

Nonetheless, the fundamental significance of Beijerinck's work is slowly becoming recognized in wider circles, and the application of enrichment culture practices is spreading. Even such up-to-date studies as those concerned with the search for antibiotics, with the attempts to culture various algae and protozoa, an endeavor so successfully pursued by Pringsheim (12), and with the selection of specific nutritional types of microbes; all such studies are now generally carried out with the conscious or unconscious inclusion of Beijerinck's principles. Furthermore, if one thinks about the reasons for the ready availability of cultures of nearly all kinds of microörganisms (yeasts, algae, actinomycetes, sulfur and hydrogen bacteria, species of *Acetobacter*, *Azotobacter*, *Aerobacter*, *Spirillum*, *Mycobacterium*, *Propionibacterium*, or *Clostridium*, lactic acid bacteria, bacteria decomposing cellulose, agar, or urea, denitrifying and sulfate-reducing bacteria, methane-producing, luminous, or photosynthetic bacteria) it becomes abundantly evident that these reasons are not to be found primarily in the existence of pure culture collections, however useful a purpose they may serve, but chiefly in the simple methodology, based on Beijerinck's enrichment culture procedures, by which these organisms can regularly be procured.

Familiarity with the results that can be achieved by means of enrichment cultures also leads to the conclusion that the distribution of diverse sorts of microbes is ubiquitous. For example, the cellulose decomposing *Cytophaga* and *Sporocytophaga* species, the nitrogen fixing azotobacters and *Clostridium pasteurianum*, or the hydrogen oxidizing bacteria that are known today can be isolated from soil, mud, or water samples in Argentina, Holland, Japan, Australia, Russia, or the U. S. with equal facility. And the pure cultures of one kind obtained in different places generally do not show any more differences among one another than do a variety of strains isolated in one and the same locality. A similar picture is presented by the microbes found in those "natural" enrichment cultures encountered in different parts of our globe, such as in hot springs, brine pools and salt beds, sulfur or iron springs. A careful comparison shows that, where the environmental conditions are closely comparable, the same types of organisms appear. The significance of these facts for determinative bacteriology is that we need not think in terms of local microfloras and -faunas. But they also carry another, less obvious implication.

There is now a large number of bacteria, yeasts, algae, and protozoa, incidentally observed under ill-defined conditions, that have been named and described on the basis of certain more or less easily ascertainable properties. Whether such characteristics, mostly determined by the application of stereotyped and arbitrary methods, bear any direct relation to those that are important in connection with the natural occurrence and survival of the organisms is often doubtful. I do not mean that it is totally useless to know that one type of photosynthetic bacteria can liquefy gelatin or grow in glucose media while others do not, or that certain fluorescent pseudomonads, in contrast to others, can utilize arabinose or produce acid from raffinose. But knowledge of such properties is entirely inadequate to tell us anything concerning the normal activities of the organisms in question. If the latter were better understood, it would become possible to approach the problems of determinative bacteriology and classification in a more rational manner, and to eliminate much of the present confusion.

For this purpose further studies with enrichment cultures are imperative. Beijerinck's great objective is still far from completed. It is necessary that conditions be more accurately controlled and specified, and that attention be paid to the effect on the outcome of enrichment cultures due to such variables as the reaction of the medium, the temperature of incubation, the concentrations of the various nutrient and non-nutrient ingredients, the presence or absence of micronutrients and growth factors, etc. It is very probable that by means of such refinements an ever increasing number of microörganisms will become accessible to isolation by enrichment culture techniques, and in this manner we shall learn more about the normal activities of the organisms encountered than by continued studies of pure cultures with standard methods. Of course, it must be admitted that such efforts may only slowly make it possible to recognize the natural environment of numerous organisms that have been isolated accidentally, and whose properties are now most imperfectly known.

When, in 1921, Beijerinck retired, a "Delft School" had been launched. The

vast knowledge of the master had in part been transmitted to his students, and some of them continued the traditions in other Dutch institutions. Also outside the Netherlands his method of approach was spreading; men like Issatchenko and Krainsky, Melin, Gran, Krzemienievski, Kaserer, Stockhausen, and Stoklasa, who had worked in his laboratory, had gone back to their own countries and served as new nuclei abroad. And yet, when we think of a "Delft School" it is certainly not only these men who come to mind. Perhaps not even, in the first place, Beijerinck, but rather his successor, Albert Jan Kluyver, Corresponding Member of our Society of American Bacteriologists, the third of the great trio of Delft microbiologists, and the scientist in whose honor today's lecture is named. By developing the concept of comparative biochemistry Kluyver laid the foundation for an approach to biochemical problems that has proved to be one of the most fruitful of our era. It has brought order into a situation that was almost chaotic, and has become the guiding principle for the study of the chemical activities of any and all living organisms.

Two years after his inauguration Kluyver (13) made a survey of the processes known to occur in nature under the influence of microörganisms. It was a bewildering picture that emerged. Not only did it show the endless variety of substances, inorganic as well as organic, that can be decomposed by bacteria, molds, yeasts, etc.; it also illustrated the enormous diversity of substances that could arise during these decompositions. Now, awareness of diversity, a prerequisite for scientific pursuit, engenders the desire to discover unifying principles. Thus the problem arose: what common denominators can be found in this multiplicity of microbial activities?

The only one apparent in 1923 was the broadest possible generalization of Lavoisier's concept of biological oxidations as the source of energy for the maintenance of life. Pasteur had extended this idea by recognizing that fermentations, *i.e.* biological processes going on in the absence of air, are also energy yielding reactions. And Winogradsky, now some 60 years ago, had discovered organisms that could fulfill their energy requirements by oxidizing inorganic compounds. When computations of energy relations showed Kluyver that the multitude of known decompositions by microörganisms all proceeded with the liberation of energy it was, therefore, clear that Lavoisier's principle in this extended form could be invoked.

But this general answer did not satisfy Kluyver. It begged the question of a mechanism. After all, these decompositions could also be considered as chemical transformations, and since the beginning of the 19th century much constructive thought had gone into making chemical reactions intelligible on the basis of the atomic and molecular theories. That it might ultimately be possible to bring the comprehension of biochemical transformations up to the same level was, consequently, a reasonable expectation.

Besides, progress had been made in this direction. The researches of Neuberg on alcoholic fermentation by yeasts had achieved an interpretation of this process as the net result of a series of consecutive step reactions, each one chemically conceivable and simple in nature. Wieland had tackled the problem of the oxida-

tion of alcohol to acetic acid by acetic acid bacteria and contended that this oxidation should be considered as composed of two stages, viz., the oxidation of alcohol to aldehyde, and of the latter—in the form of a hydrate—to acetic acid. Both these oxidations appeared to be reactions in which two hydrogen atoms are eliminated from a substrate molecule and transferred to any one of a number of hydrogen acceptors, such as O_2, quinone, methylene blue, etc. From these results Wieland had drawn the conclusion that all biological oxidations could be interpreted as primarily composed of series of dehydrogenations, with O_2 acting as the normal, but not the only possible H-acceptor. Harden and his coworkers, especially Grey, had made a good beginning with the resolution of the coli and aerobacter fermentations; and Fred and Peterson, as well as Speakman, of the butanol-acetone fermentation.

It would take too long to review the developments that led Kluyver, in a few years, to the masterly syntheses represented by the two major publications: "The unity in biochemistry" (14), and "The chemical activities of microörganisms" (15). It is in the latter treatise that the term "comparative biochemistry" was first used, and Kluyver envisaged for it an influence which could benefit biochemistry in a manner similar to that in which the concept of "comparative anatomy" had helped to bring order into the mass of isolated anatomical observations.

Kluyver's keen and critical mind recognized the potential significance of the ideas that Neuberg, Wieland, Warburg, Harden, and a few others had advanced to account for more or less specific biochemical events. Soon it became evident to him that those concepts could be welded together into a very few general principles, applicable to all biochemical phenomena. The most basic of these generalizations is the extension of the ideas of Neuberg and of Wieland to their ultimate limits. Thus, any biochemical process, whether oxidation, fermentation, or synthetic reaction, was considered as a chain of step reactions, each one of which represented a simple mechanism in which hydrogen is transferred from one molecule, the H-donor, to another, the H-acceptor. The only apparent exception to this principle was exhibited in the metabolism of complex molecules, composed of a number of simple entities, for example the polysaccharides (complexes of simple sugars), proteins (complexes of amino acids), and fats (complexes of fatty acids and glycerol). Such complexes would first be converted to their constituent units by hydrolytic cleavages, with the products subsequently undergoing the various hydrogen-transfer reactions. In this manner the existence of the many hydrolytic enzymes—glucosidases, proteinases, lipases, etc.—could be fitted into the general picture.

Many of the known facts concerning diverse metabolic processes could be readily incorporated into this concept. In the course of the following years numerous additional cases were investigated in his laboratory, and the outcome of this activity did much to strengthen the evidence for the soundness of the postulates. It also indicated that the initial stages in the biochemical transformations of a specific substance were very similar, if not identical, no matter what the final result proved to be. For example, the evidence strongly suggested the

probability that in practically all instances of sugar decomposition the carbohydrate would first be degraded to three-carbon moieties; the differences in the end products reflected differences in the fate of these universal intermediate products. It is important to realize that the arguments applied to a great diversity of processes, such as the alcoholic and lactic acid fermentations, the "mixed acid" fermentation characteristic of the coli group, the butane-diol fermentation of *Aerobacter* and *Aerobacillus* species, the propionic acid fermentation, the butyric acid and the butanol fermentations, the fermentations in which acetone and isopropanol are produced. Also, many of the oxidative degradations appeared to proceed by the same initial stages.

It would be foolish to insist that the principles of comparative biochemistry would not have been developed if it had not been for Kluyver's penetrating approach, just as it would be foolish to contend that microörganisms would not have been discovered if Antonie van Leeuwenhoek had not done so. In the late twenties there were others who were beginning to think along similar lines, and the reconciliation of Wieland's and of Warburg's ideas on the nature of biological oxidations was proposed almost simultaneously by Kluyver and Donker, Szent-Györgi, and Fleisch, in three entirely independent publications. Nevertheless, the familiarity with the vast diversity of the conditions under which life can exist and manifest itself, especially in the world of microörganisms, made available for Kluyver's scientific contemplation an immensely greater range of patterns than that presented by the higher plants and animals. And the result was the enunciation of the most far-reaching generalization.

The attempts at interpreting various biochemical phenomena in greater detail led Kluyver and his collaborators to postulate a number of specific step reactions, leading to a small group of common intermediate products. It was clearly recognized that some substrates or intermediates could undergo more than one particular conversion. The extent to which each of the possible transformations occurs would, of course, depend on the nature of the organisms, *i.e.*, its enzymatic composition. But even for the same organism the result is usually not fixed because environmental conditions, such as temperature, concentration of substrate or intermediate products, reaction of the medium, the presence or absence of special hydrogen donors or acceptors, could readily influence the magnitude of the different conversions. It is, therefore, impossible to predict the exact outcome of a biochemical process in terms of the precise quantities in which each of the end products will be formed. The frequently observed fluctuations in this respect need not be disturbing, however; they become readily understandable as the result of a complicated interplay between the various potentially possible reactions in which the intermediate products can participate. When viewed in this manner a biochemical reaction becomes more clearly a dynamic event, to be represented by series of steps with variations in several directions rather than by a single chemical equation with fixed quantitative relations between the end products.

Many of the step reactions and intermediate products postulated by Kluyver some twenty years ago appear outmoded to-day. Surely no biochemist would now seriously consider methyl glyoxal, for example, in the central position which

Kluyver assigned to it in his London Lectures (15). Much has been accomplished in the intervening years through the brilliant work of many scientists. The chemical nature of several intermediate products has been established with increasing precision; the interactions and conversions of these compounds can now be represented by reaction chains far more elaborate than was once deemed possible. In large part this astounding penetration into details of biochemical mechanisms has resulted from the isolation of specific enzymes with which partial conversions can be investigated under rigorously defined conditions. And much of this work has been done with microörganisms; those who have attended the symposium on the first day of our meetings will realize this.

Furthermore, new principles have been introduced. Among the most important ones must be mentioned Michaelis' theory (16) of the single-electron shifts; Lipmann's concept (17) of the high-energy phosphate bond and its significance for the preservation and storage of energy; and the ideas concerning the transfer of whole blocks of atoms, as in trans-aminations, trans-methylations, trans-acetylations, trans-glucosidations and trans-phosphorylations. It has been a phenomenal development. But, although these advances have shown the need for modifying the earlier postulated details, they have also served to substantiate the validity of Kluyver's main thesis regarding the fundamental unity in biochemistry. The basic similarity in the biochemical behavior of so many different organisms is now generally admitted. It is emphasized by the occurrence of the same amino acids, vitamins, enzymes, etc., in all forms of life, and by the participation of a number of identical intermediate products in practically all metabolic activities.

The recognition of this unity is Kluyver's great contribution; it is also the starting point of "comparative biochemistry". Predicated upon the fact that a particular substance, whether substrate or intermediate product, can undergo only a limited number of immediate transformations, sometimes only a single one, these can be explored by investigating the fate of such compounds under the influence of different organisms. The results so far obtained have amply demonstrated the fruitfulness of this line of study.

A good example is furnished by the methane fermentation, a process in which various alcohols and fatty acids are decomposed to methane, generally accompanied by the production of carbon dioxide. Now, the primary attack on those substrates cannot readily be conceived of as anything but a straight dehydrogenation. Hence an external hydrogen acceptor is required. Decompositions of the same substrates are known to occur in the presence of oxygen, nitrate, and sulfate, and these substances, acting as hydrogen acceptors, are thus converted into H_2O, H_3N, or H_2S respectively. This led to the idea that the methane fermentation represents a similar substrate oxidation with CO_2 as hydrogen acceptor, a postulate for which the investigations of Barker (19) have furnished experimental evidence. The degradation of the higher fatty acids during the methane fermentation has been shown by Mrs. Stadtman (personal communication) to follow exactly the path required by the Knoop-Dakin theory for this process in higher animals. Hence the methane fermentation no longer occupies a totally unique

position. The details of the mechanism whereby carbon dioxide is reduced to methane remain to be elucidated, and these might yield important results for an understanding of the mechanism of photosynthesis.

I realize that this may seem a far-fetched conclusion. However, the following considerations, in exposing the trend of thought upon which this deduction rests, should make it appear reasonable. A comparison between the photosynthetic activities of green plants and of green and purple bacteria suggested, several years ago, that photosynthesis should be interpreted as a process of carbon dioxide reduction with hydrogen obtained by a photochemical decomposition of water (20). This, in turn, implies that the reactions more immediately concerned with the assimilation and reduction of carbon dioxide must themselves be non-photochemical processes. It should consequently be possible to reach a better understanding of the essential features of these reactions by a comparative study of all cases in which carbon dioxide is similarly involved. And those include not only the carbon dioxide assimilation by chemo-autotrophic microbes, but also the Wood and Werkman reaction (21), the formation of other di- and tricarboxylic acids by carbon dioxide addition to various keto-compounds (22), the production of acetic acid from CO_2 and hydrogen by *Clostridium aceticum* (23), and the methane fermentation. Such a comparative study would make it possible to discover the common denominators of all these processes, and therefore contribute to a more detailed picture of the photosynthetic reaction.

Two decades ago Kluyver advocated the use of microörganisms for comparative biochemical studies. On several occasions he stressed the advantages they offered, both on account of the ease of handling them under controlled and reproducible conditions, and because of the enormous biochemical versatility encountered within this group. It is often possible to select a specific microörganism as singularly appropriate for a given problem because it carries out a certain type of reaction to the exclusion of almost any other. But it is equally important to realize that one may find among these creatures the best examples of seemingly quite different biochemical properties with respect to the conversion of a particular substrate. Both of these aspects are important for a comparative biochemical approach. If it be further remembered that by the application of Beijerinck's principle of enrichment cultures many of the organisms are so readily procurable, it will be clear that the case for the microbes—and for the microbiologist—is pretty strong.

This has obviously been recognized. During the past decade there has been a rapidly growing interest in comparative biochemistry as well as in microörganisms. It is no longer unusual to find a large fraction of the pages of physiological and biochemical journals occupied by publications dealing with the activities of fungi, protozoa, and bacteria. Even in the field of genetics the mold Neurospora, the yeasts, *Escherichia coli*, Paramecium, and bacteriophages are successfully competing with Oenothera, *Zea mais*, and Drosophila.

When nowadays enzyme reactions are studied by methods ranging from kinetic measurements (24, 25) to the use of mashed cells, of dried cell preparations (26), of cultures supplied with sub-optimal amounts of growth factors (27–30), of in-

duced mutations (31), of anti-vitamins (32), or of adaptive enzyme systems (33), one finds that microörganisms are used in the majority of cases. But it must be realized that this is generally done with the tacit implications that the results will be of importance for a better understanding also of similar processes occurring in other organisms. This attitude has been amply justified. The earlier remarks concerning the mode of degradation of higher fatty acids in the methane fermentation and in the animal body provide a good example. The recent studies of Heidelberger *et al.* (34, 35) on the decomposition of tryptophan in mammals have shown that this is accomplished by a mechanism which appears to be identical with that previously demonstrated in the mold Neurospora. It will be superfluous to elaborate this theme any further.

The increased interest in general microbiology is apparent also in other ways. It is no longer necessary for the confirmed microbiologist to feel that he supports a worthy cause in vain when he expresses the devout wish that those responsible for the development of science in colleges and universities might eventually "see the light", and establish positions for teaching and research in this field. Surely, general microbiology is now a rapidly expanding science, and I firmly believe that it is an easily defensible thesis to propose that its spectacular rise is due in large part to the Delft School. However, the encouragement by our institutions of higher learning might also have another cause. Some of the interest displayed could have been stirred up by an overemphasis on developments of the past decade resulting from studies on vitamins, chemotherapy, and antibiotics. These researches have been well publicized, and rightly so, because the advances made have been striking, and the applications both numerous and successful. Nevertheless, these topics represent only a small segment of the field of general microbiology, and I hope that the other aspects will not be neglected.

That Albert Jan Kluyver was chosen as the microbiologist who is to be honored to-day bears convincing witness to the fact that our Society of American Bacteriologists is concerned with the broad principles. For the work of the "Delft School" carries implications of deep philosophical importance that must appeal to any one who is still willing to subscribe to Ernest Renan's dictum: "Le but du monde, c'est l'Idée."

Beijerinck's major contributions can be considered as the first direct experimental investigations of Darwin's principle of natural selection. In the enrichment cultures the experimentally defined environmental conditions are the selecting agent, and the outcome of the cultures can provide an unambiguous answer to the question as to what organisms among the many types present in the inoculum are most fit to cope with the environment. This having been established by the "endless experiments", one can even try to penetrate further, and determine the mechanism by which the selection operates.

So far, it can be stated with some assurance that the significant factors are physical (light, temperature, concentrations) and chemical in nature. In many cases it is obvious that those organisms whose minimum nutrient requirements are fulfilled by the culture medium will come to the fore. This, together with other cases of successful competition, operating through the production by one

kind of organism of substances which inhibit or prevent the development of other competitors, constitutes strong evidence in support of the idea that ecology, at least as far as microörganisms are concerned, rests principally on a biochemical basis.

The significance of biochemistry for a better understanding of the behavior of living organisms is further attested to by the modern trends in genetics. Nearly all the studies in this field of physiological or biochemical genetics are carried out with microbes, and most of these investigations are patterned on the important work of Beadle and Tatum and their collaborators. What has come out of the numerous contributions, in which algae, molds, yeasts, protozoa, bacteria, and phage play so important a part, supports an idea expressed as early as 1917 by Beijerinck (36), namely, that genetic characters function by way of controlling the formation of enzymes. There is now a wealth of information in favor of the supposition that one genetic character is involved in the control of a single enzyme. A by-product of these studies has been the use of genetically modified strains for the successful elucidation of the detailed mechanism of biochemical syntheses.

But there are also many examples known in which environmental rather than genetic factors influence directly the enzymatic composition of microörganisms. These are, of course, the numerous cases of adaptive enzyme formation in which the presence of a particular substrate elicits the formation of a corresponding enzyme system capable of catalyzing the transformation of the substrate in question. It is possible to submit that in such instances the genetic constitution of the organism confers upon it the potentiality of responding to an environmental stimulus. However, the common interpretation of the one gene-one enzyme concept does not generally connote such a degree of flexibility, and it will be interesting to see how the ideas on the fundamentals of genetics will, in the course of time, be modified so that they can account for the determinative effect of external factors.

These phenomena of adaptation bring into sharp focus the fact that microbes, like human beings, are subject to and respond to environmental influences, and do not necessarily represent rigidly determined systems. This adaptability, together with the frequent spontaneous mutations exhibited by living organisms, emphasizes their innate variability. In another, perhaps more basic sense, there is no denying the existence of a high degree of constancy. These two aspects of life—its constancy and variability—are reflected in many ways. From the point of view of comparative biochemistry, the constancy finds its expression and counterpart in the unity of the fundamental biochemical mechanisms, that is, Kluyver's concept of the "unity in biochemistry." This, to-day, is also the most compelling argument in favor of a monophyletic origin of life. The variability, by comparison, can be related to the existing biochemical diversity, so glaringly apparent especially among microörganisms, and it represents the numerous directions in which adaptations to a new environment have become established. The persistence of so many patterns, like variations of a theme, drives home the importance of individuality, without which there could be no differences—nor evolution.

And I hope that you may be found willing to consider seriously the proposition that an important aspect of evolution consists in the acquisition of increased comprehension. Comprehension not for the sake of power—there is too much of that in the hands of too few—but for the sake of a possible evolution of man to a state in which he is no longer at war with himself and his contemporaries, no longer at odds with nature, but an integral part of it. The implication of this is the need for recognition of the intrinsic value of the individual as the unique, potential step towards something new and better. If this is appreciated we shall also have gone far in understanding the great significance of another phase of the profound influence wielded by the founder of the "Delft School". For Albert Jan Kluyver has been a living example of this attitude towards the individual. Those who have had the great good fortune of experiencing his influence—and there are many of them among my audience—can never be grateful enough.

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A MECHANICAL KEY FOR THE GENERIC IDENTIFICATION OF BACTERIA

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THE NEED FOR ADEQUATE DESCRIPTIONS

Many of the descriptions of bacteria in Bergey's Manual of Determinative Bacteriology (1939, 1948) are decidedly poor when viewed from present-day standards. Some will be difficult to improve since a number of the original cultures have probably been lost. The original descriptions which still remain on record present us with an awkward problem in establishing priorities. Some of these descriptions are so inadequate that one description could be equally well applied to many new isolates. The original authors cannot be blamed for the inadequacy of these descriptions which no doubt conformed to the standard of the day and it would be a breach of ethics to refuse recognition of these descriptions. Nevertheless present-day workers cannot regain the original cultures in some instances to subject then to further examination and would-be key formers are handicapped by the lack of this information. Thus one cause of the chaotic state of bacterial nomenclature is the lack of "type" specimens regarded as essential by systematic botanists. There is only one remedy for this, namely the redescription of all available cultures according to a certain code which should be applied to all bacteria alike. On the basis of these descriptions the organisms should be renamed, for the most part with the names they now possess. Priorities should be based on these names and all descriptions and names for which there are no procurable cultures should, by common consent, be discarded.

Such a move would necessarily have to be backed by a definite decision on a code for description and standardization of methods whereby characters employed in the code are determined. Although an attempt has been made on an international basis to maintain uniformity in methods of nomenclature, no attempt has been made to standardize the methods by which tests are carried out to determine the characteristics of a species. The Society of American Bacteriologists through its Committee on Bacteriological Technic has suggested a scheme whereby all organisms should be described and in its "Manual of Methods of Pure Culture Study of Bacteria" has made an attempt to standardize the methods by which the tests are carried out. The scheme is considered by some to be too extensive and quite unnecessary for some groups of organisms. I cannot agree with this attitude. It is easier to discard evidence considered unnecessary than it is to repeat an investigation through lack of it.

A descriptive code need not be static. It could be expanded or contracted as the need arises. Expansion would necessitate further investigation of described species. Necessary alterations can easily be made to mechanical keys.

A uniform code should be enforced and publication of descriptions should be contingent on complete descriptions being submitted, even if this entailed work-

ers in poorly equipped laboratories having to submit cultures to recognized institutions for complete description. The main objection to this procedure would be overcome if the worker submitting the culture retained the right of discovery and the right to name the organism.

THE DEFINITION OF SPECIES AND GENERA

A species is defined only by those characteristics which, through their constancy and stability have served in the past to assign it to a genus, together with those characteristics which have served to separate it from other species in the same genus. A distinct advantage would be gained if a list of these characteristics were appended to the name of each bacterium prior to the general description. Attention would then be focused on these essential characteristics and a better appreciation of the extent and importance of variations be gained.

Characters which are not employed in differentiation of the species could be employed for the subdivision of species into types.

There is also need for more precise definitions for genera. In the hands of the authors of most of our textbooks the term "definition" has entirely lost its meaning. Many of the definitions contain very little which is definite. They approach more towards condensed, and often confusing, descriptions which attempt to embrace all the possibilities which one may encounter among the species in the genus rather than a precise statement of the characters which can be uniformly found among all or the majority of species within that genus and on the basis of which one might expect the genus to be distinguished from other genera.

Let us take for example the definition of the genus *Streptococcus* from Topley and Wilson's Principles of Bacteriology and Immunity (1947). It reads as follows:

DEFINITION. *Streptococcus.* (Author's italics.)

Spherical or ovoid cells, arranged in short or long chains or in pairs. *Usually* non-motile. Non-sporing. *Most* species Gram-positive. *Some* species form capsules. Growth *tends to be* relatively slight on artificial media and *some* species grow poorly in the absence of native protein. *Several* species produce characteristic changes in media containing blood. *Various* carbohydrates are fermented with the production of acid. *Most* species fail to liquefy gelatin. *Most* species are aerobic and facultatively anaerobic. *Some* are anaerobic. *Many* species are normally pathogenic on man or animals; *some* species are highly pathogenic and *some* produce soluble toxins.

This so-called "definition" contains two statements, the first and third, which can reasonably be applied to all species and therefore could be said to characterize the genus if the genus *Leuconostoc* is not recognized.

The above "definition" would be more aptly termed a "description."

There is a great need for precise definitions in systematic studies. Adoption of precise definitions for species should automatically lead to more precise definition of genera to which they belong and the characters used in the definition of genera would, like those used in defining species, be definitely determinable. Inclusion of species in genera on purely speculative ground should be entirely eliminated. It would be infinitely better if a new genus was created for species which do not

conform to the general pattern, particularly if one views the genera as "form genera" without any phylogenetical significance.

Variation will no doubt be raised as a barrier to this unquestionably idealistic approach to bacterial classification. In the opinion of some workers variation is so prevalent that definition of species would be impossible. But despite this we cannot lose sight of the fact that over the past half century a great number of organisms have been described and have definitely attained species rank. Recognition of them as species has come through the gradual recognition of the constancy of certain characteristics which they display. Variants have been recognized only because certain characters were selected as representative of a species, that is, a certain standard was created. It is a fundamental fact that without such standards there can be no variants, otherwise all variants would themselves assume the status of standards. For this reason the acceptance of a suitably worded list of the stable characters of a species as a "definition" for the species is entirely justified.

THE CLASSIFICATION OF BACTERIA; FORMATION OF HIGHER GROUPS

The adoption of the botanical system of nomenclature made possible the segregation of bacteria into Species, Genera, Tribes, Families and Orders. A number of classifications have been suggested. These have been reviewed by van Niel (1946) and have been discussed briefly in Bergey's Manual. Van Niel questions the wisdom of attempting to form a "classification" on the evidence which is at present available but in so doing he does not discount the fact that there is strong evidence for phylogenetical relationships between some groups of bacteria. A close study of the number of determinable characters which could represent all species within each genus would reveal this number to be very small. The number of characters which are common to all genera within a Tribe must inevitably be smaller, and would continue to diminish as groupings become broader. Where groupings have become impossible on purely determinable characters resort has to be made to the use of certain assumptions which originate from the study, not of single organisms, but of large groups of organisms. On the basis of these assumptions some bacteria may be placed in higher groupings on a character or characters which they do not possess but which one is led by assumption to believe that they probably did possess by virtue of obvious relationships to other bacteria which do possess these characters. This was the case with *Corynebacterium pseudodiphthericum* (hofmanni) which in the fifth edition of the Manual was placed in the Order *Actinomycetales*, because of its relationship to certain other species in the same genus reputed to show positive evidence of branching—a salient character of the Order *Actinomycetales*. But how one was expected to identify the organism by first determining a character which it did not possess is difficult to understand.

This is the obvious result of an attempt to form a key for *identification* which operates in the reverse direction to that in which the *classification* was constructed. In the development of our system of classification the discovery and naming of species with a generic and specific name came first. Grouping into

Genera was followed by grouping of Genera into Tribes and Tribes into Families and Families into Orders. In developing the key in the reverse order, the authors of the keys in the Manual were forced to use initially for identification characters which by their very nature are largely indeterminable.

Van Niel struck at the heart of this matter and advocated the complete separation of a system of identification from a system of classification. He suggested the formation of a number of mechanical keys for the identification of bacteria. I am strongly in favor of van Niel's suggestion. It is the only way to overcome the vexatious problem of identifying organisms. Van Niel did not elaborate greatly on the type of keys he envisaged. I therefore suggest that initially keys should be formed for the identification of species into genera. Subsequent keys could be formed for identification of species in each genus on the lines already followed in the Manual. Concurrent with the production of generic keys and keys to species for purposes of identification, systematists could also direct attention to the formation of keys to *show how* Tribes are formed from Genera, Families from Tribes and so on. Such keys should assume a different form from those used for identifying bacteria and should be largely discussional. They should *aim to educate* all workers in this fascinating field of biological relationships.

A key to the genera of bacteria is presented in this paper. Genera included are those in the Suborder *Eubacteriineae* of the Order *Eubacteriales* and those in the Order *Actinomycetales* in the sixth edition of Bergey's Manual of Determinative Bacteriology. For the convenience of all workers, the generic terminologies employed in the fifth and sixth editions of the Manual and that employed in the third edition of Topley and Wilson's "Principles of Bacteriology and Immunity" have all been included. The only genus omitted is the miscellaneous genus *Bacterium* as used in the Manual.

The bacteria in the Suborder *Eubacteriineae* are characterized by the possession of rigid cell walls, the absence of photosynthetic pigments and the absence of a stalk which would fix them to their substrate. Those in the Order *Actinomycetales* are similar except that they show marked or rudimentary branching. Unfortunately not all species included in the Order do branch and the absence of the character renders their identification difficult. For this reason all species in the order have been treated with those in the Suborder *Eubacteriineae* in the key.

It must be emphasized that the only advantage that can be claimed for the key is that it is workable. It cannot improve in any way on the original material. It was quite apparent at the outset that it was impossible to obtain the type of "definition" for individual genera advocated in the earlier part of this paper. It was impossible to obtain a combination of characters for every genus which would separate it from other genera. Therefore in preparing the key the only other course was adopted. The reactions of all bacteria given species rank in the Manual were tabulated and characters were selected which appeared to be uniform for each separate genus. In some instances this was found impossible and genera had to be subdivided and each subdivision treated separately. In some instances descriptions from Topley and Wilson's "Principles of Bacteriology and Immunity" were substituted for those in the Manual.

Characters employed in the key were selected solely on their suitability for separating organisms and the ease with which they could be determined. No other importance was attached to any of the tests. As far as possible an attempt was made to keep the sequence of characters in line with accepted laboratory practice.

Quite frequently information on the reaction of species to tests employed in the key was not available. In such cases the species has been treated as being either positive or negative and separation has been effected in both sections of the key. The same procedure was adopted where species were known to be variable within a genus to a particular character, e.g., with motility in *Escherichia coli*. The absence of information has greatly increased the length of the key. It could be considerably reduced in size if the information was made available.

The key is of a type commonly employed by botanists. The numbers on the left hand side of the key cover, for the greater part, pairs of opposing characters such as "gram positive or gram negative." The character of the pair which applies to the organism in question is selected and the number immediately on the right indicates the next number on the left to be consulted. The numbers must be followed in strict sequence until a number on the right is replaced by a generic name. A single example is given.

For species of the genus *Escherichia* the number sequence is as follows: 1–7; 7–10; 10–44; 44–45; 45–46; 46–47; 47–48; 48–49; 49–50; 50–52; 52–54; 54–57; 57–62; 62–63; 63–64; 64–65 or 64–71

65–66; 66–67; 67—*Escherichia;*
or, 71–72; 72–73; 73—*Escherichia.*

THE KEY

1. Organisms which can grow on a mineral salts medium with carbon dioxide as the sole source of carbon; strict autotrophs which will not grow on meat extract agar; do not oxidize hydrogen to water 2
   Other than above 7
2. Organisms which oxidize ammonia to nitrites 3
   Organisms which oxidize nitrite to nitrate 6
   Organisms which oxidize sulfur compounds (inorganic) *Thiobacillus*
3. Zoogloea formed 4
   Zoogloea not formed 5
4. Zoogloea surrounded by a membrane, forming a cyst . . . . . *Nitrosocystis*
   Zoogloea not encysted . . . . . *Nitrosogloea*
5. Cells ellipsoidal . . . . . *Nitrosomonas*
   Cells spherical . . . . . *Nitrosococcus*
   Cells spiral . . . . . *Nitrosospira*
6. Zoogloea present . . . . . *Nitrocystis*
   Zoogloea absent . . . . . *Nitrobacter*
7. Organisms will grow on a mineral salts medium with carbon dioxide as a sole source of carbon but will also grow on meat extract agar 8
   Organisms will grow in a mineral salts medium if carbon monoxide or methane is present as a sole source of carbon 9
   Other than above 10
8. Organisms grow autotrophically oxidizing hydrogen to water . . . . . *Hydrogenomonas*
   Organisms grow autotrophically oxidizing sulfur compounds . . . . . *Thiobacillus*
9. Organisms will grow autotrophically using carbon monoxide as the sole source of carbon . . . . . *Carboxydomonas* (5th ed.)

(Note: the genus *Carboxydomonas* has been appended to the genus *Streptomyces* in the 6th edition of the Manual.)

Organisms will grow autotrophically using methane as a source of carbon and energy ..... *Methanomonas*

10. Gram positive 11
    Gram negative 44
11. Organisms will grow in contact with air without the addition of specific reducing substances to the medium 12
    Organisms will grow only in the absence of air, or in the presence of air if specific reducing substances are added to the medium 37
12. Rods and cocco-bacilli 13
    Cocci 33
13. Sporing rods ..... *Bacillus*
    Non-sporing rods 14
14. Organisms showing distinct branching 15
    Organisms not branched 17
15. Long intertwining branching forms showing little or no tendency to fragmentation of the pseudomycelium except at the tips where short conidia-like bodies are formed in large numbers in chains ..... *Actinomyces* (5th ed.)
    Streptomyces (6th ed.)
    Actinomyces (T & W.)
    Similar to above except that only one "conidium" is produced at the end of each side branch or "conidiophore" ..... *Proactinomyces* (5th ed.)
    Micromonospora (6th ed.)
    Actinomyces (T & W.)
    Branched rods which do not produce conidia 16
16. Organisms present in young cultures as branched filaments which rapidly disintegrate to produce short branched rods and some unbranched rods and coccal forms; non-acid fast or only weakly acid fast; obligate aerobes ..... *Actinomyces* (5th ed.)
    Nocardia (6th ed.)
    Actinomyces (T & W.)
    Branched rods with little or no tendency to form filaments; colonies dry and crumbly; acid fast ..... *Mycobacterium*
    Branched rods with only a slight tendency to form filaments; division by a sharp snapping action resulting in palisade formation; non-acid fast ..... *Corynebacterium*
17. Motile rods 18
    Non-motile rods 22
18. Carbohydrate fermented 19
    Carbohydrates not fermented ..... *Kurthia* (5th ed.)
    Zopfius (T & W.)
    placed in genus *Bacterium* (6th ed.)
19. Organisms pathogenic to plants 20
    Organisms not pathogenic to plants; pathogenic to warm-blooded animals causing monocytosis ..... *Listerella* (5th ed.)
    Listeria (6th ed.)
    Erysipelothrix (T & W.)
20. Yellow pigmented colonies ..... *Xanthomonas* (6th ed.)
    Colonies with no yellow pigments 21
21. Flagella polar ..... *Phytomonas* (5th ed.)
    Flagella peritrichiate ..... *Erwinia*
22. Unbranched rods; colonies dry and granular; organisms have twisted axes and tend to form in clumps; acid fast ..... *Mycobacterium*
    Not as above; non-acid fast 23

23. Litmus milk acid or acid and coagulated 24
Litmus milk alkaline or unchanged 29
24. Litmus milk acid and coagulated 25
Litmus milk acid only 26
25. Organisms beaded and club shaped; pleomorphic; pathogenic to guinea pigs, rabbits and mice; no growth in a glucose acetic acid broth with pH 4.5 *Corynebacterium*
Non-pleomorphic; non-pathogenic; growth in glucose acetic acid broth with pH 4.5 *Lactobacillus*
26. Colonies small, dewdrop, about 0.2 mm in diameter; in smooth colonies the organisms appear as short rods; in rough colonies they appear as long filaments; no growth in acetic acid glucose broth with pH 4.5; cause erysipelas in man and animals *Erysipelothrix*
Not as above 27
27. Catalase positive 28
Catalase negative . . . . . *Lactobacillus*
28. Small thin rods; granular staining; resist heating to 71 C for 2½ minutes *Microbacterium*
Not as above . . . . . *Corynebacterium*
29. Growth in acetic acid glucose broth at pH 4.5; non-pleomorphic rods producing over 1% lactic acid from glucose without continuous neutralization . . *Lactobacillus*
Not as above 30
30. Human mouth parasites; long tangled threads intermingled with a few short rods *Leptotrichia* (5th ed.)
appended to genus *Lactobacillus* (6th ed.)
Not as above 31
31. Small dewdrop colonies; organisms cause erysipelas in man and animals *Erysipelothrix*
Not as above 32
32. Rods straight or curved; arranged singly; non-pleomorphic; litmus milk alkaline and peptonized; no carbohydrates fermented . . . . . *Alcaligenes*
treated in the miscellaneous section by T & W.
Rods usually in palisades; division by a characteristic snapping motion; pleomorphism common; litmus milk may be rendered acid or alkaline but is not peptonized *Corynebacterium*
33. Organisms arranged in cubical packets . . . . . *Sarcina*
Organisms arranged in chains or in diplococci in liquid media 34
Organisms arranged in clusters other than cubical packets 36
34. Kidney shaped or hemispherical organisms arranged in diplococci in clumps. Colonies high, convex, and granular and usually difficult to emulsify; species cause gonorrhea, meningitis; others normal inhabitants of the throat . . . . . *Neisseria*
Spherical or oval organisms in chains or in diplococci which do not form clusters 35
35. Organisms producing a slimy pellicle in sucrose broth; in which the organisms are heavily capsulated and form zoogloea . . . . . *Leuconostoc*
Other than above . . . . . *Streptococcus*
including the genus *Diplococcus*
36. Organisms divide in two directions producing plates of organisms all lying in the one plane . . . . . *Staphylococcus* (5th ed.)
Micrococcus (6th ed.)
Staphylococcus (T & W.)
including the genus *Gaffkya*
Organisms apparently divide in more than two directions producing clusters or organisms lying in more than one plane . . . . . *Micrococcus*
37. Cocci 33
Rods 38

38. Branching rods 39
Not branching rods 40
39. Long branching filaments which show no tendency to disintegrate; produce a single "conidium" at the ends of short side branches or "conidiophores"
Actinomyces (5th ed.)
Micromonospora (6th ed.)
Actinomyces (T & W.)
Long branching filaments showing no tendency to disintegrate except at the tips of aerial "hyphae" where chains of "conidia" are produced; giving the colony a dry powdery surface ..... *Actinomyces* (5th ed.)
Streptomyces (6th ed.)
Actinomyces (T & W.)
Short branching rods. In very young cultures branching filaments are formed which rapidly disintegrate to form short branched rods and some unbranched rods and cocci; no conidia formed ..... *Actinomyces* (5th ed.)
Actinomyces (6th ed.)
Actinomyces (T & W.)
40. Sporing rods ..... *Clostridium*
Non-sporing rods 41
41. Plant pathogens; motile 42
Not plant pathogens; non-motile 43
42. Flagella peritrichiate ..... *Erwinia*
Bacterium (T & W.)
Flagella polar ..... *Phytomonas* (5th ed.)
43. Catalase positive; propionic acid produced from lactic acid and from glucose
Propionibacterium
Catalase negative; butyric acid produced from lactic acid and from glucose
Butyribacterium (6th ed.)
Catalase negative; lactic acid accumulates as an end product of glucose breakdown
Lactobacillus
44. Organisms grow in contact with oxygen without the addition of specific reducing substances to the medium 45
Organisms will not grow in contact with oxygen unless some specific reducing substances are added to the medium 118
45. Organisms isolated from and capable of producing nodules on the roots of leguminous plants ..... *Rhizobium*
Not as above 46
46. Organisms capable of continued growth in a medium devoid of inorganic or organic nitrogen compounds; large rod-shaped organisms which change to spherical forms in ageing cultures ..... *Azotobacter*
Not as above 47
47. Kidney shaped or hemispherical organisms arranged in pairs in clusters; organisms found commonly in the throat ..... *Neisseria*
Rods and cocco-bacilli 48
48. Sporing rods ..... *Bacillus*
Non-sporing rods 49
49. Organisms grow well in a yeast water medium containing up to 10% alcohol, oxidizing the latter to acetic acid ..... *Acetobacter*
Not as above 50
50. Colonies with a purple pigment ..... *Chromobacterium*
Colonies with a red pigment 51
Colonies other than the above 52
51. Motile rods; do not attack alkylamines ..... *Serratia*
Chromobacterium (T & W.)
Motile rods capable of attacking alkylamines ..... *Protaminobacter*

52. Organisms producing water soluble green, blue, or yellow pigments which diffuse into the medium 53
Not as above; pigments, if produced, are not water soluble 54
53. Plant pathogens ..... *Phytomonas* (5th ed.)
Pseudomonas (6th ed.)
Not plant pathogens ..... *Pseudomonas*
54. Organisms isolated from disease lesions in plants 55
Not as above 57
55. Yellow pigment produced which is not soluble in water ..... *Phytomonas* (5th ed.)
Xanthomonas (6th ed.)
No yellow pigment produced 56
56. Flagella polar ..... *Phytomonas* (5th ed.)
some species in the the genus *Agrobacterium* (6th ed.)
other species apparently in the genus *Pseudomonas* (6th ed.)
Flagella peritrichiate ..... *Erwinia*
Bacterium (T & W.)
57. Curved rods, single or united in chains 58
Straight rods and cocco-bacilli 62
58. Rods curved; organisms capable of using phenolic compounds as a source of carbon; gas produced in 0.1% nitrate broth but no nitrites are produced; no carbohydrates fermented ..... *Mycoplana*
Not as above 59
59. Short stout rods, motile with a single flagellum 60
Long thin rods 61
60. Cellulose oxidized to oxycellulose; no growth on meat infusion agar; cells curved with pointed ends ..... *Cellfacicula*
Cellulose is not oxidized ..... *Vibrio*
Note: The genus *Thiospira* (6th ed.) is not treated.
61. Motile with a single flagellum; cellulose oxidized ..... *Cellvibrio*
Motile with several polar flagella; cellulose is not oxidized ..... *Spirillum*
(except *S. virginianum*)
62. Lactose fermented within seven days 63
Lactose not fermented within seven days 84
63. Growth on MacConkey's agar in three days at 37 C 64
Not as above 76
64. Motile at 37 C 65
Non-motile at 37 C 71
65. Acid and gas produced from glucose 66
Acid only produced from glucose 68
66. Colonies yellow ..... *Flavobacterium*
Chromobacterium (T & W.)
Colonies not pigmented 67
67. Methyl red positive; Voges Proskauer negative ..... *Escherichia*
Bacterium (T & W.)
Methyl red negative; Voges Proskauer positive ..... *Aerobacter*
Bacterium (T & W.)
68. Indole produced ..... *Eberthella* (5th ed.)
appended to the *Salmonella* (6th ed.)
Indole not produced 69
69. Small translucent colonies; greyish yellow; mucoid; small slender rods in smooth colonies; ovoid rods with bipolar staining in rough colonies; cause melioidosis in man and animals ..... *Malleomyces*
Pfeifferella (T & W.)
Not as above 70

70. Yellow pigmented colonies ..... *Flavobacterium*
Chromobacterium (T & W.)
Colonies not pigmented ..... *Achromobacter*
71. Acid and gas from glucose 72
Acid only from glucose 74
72. Methyl red positive; Voges Proskauer negative 73
Methyl red negative; Voges Proskauer positive ..... *Aerobacter*
Bacterium (T & W.)
73. Colonies not mucoid; organisms not capsulated ..... *Escherichia*
Bacterium (T & W.)
Colonies mucoid; organisms capsulated; pathogenic causing throat infections in man and animals ..... *Klebsiella*
Bacterium (T & W.)
74. Yellow pigmented colonies ..... *Flavobacterium*
Chromobacterium (T & W.)
Colonies not pigmented 75
75. Enteric organisms; optimum temperature 37 C; pathogenic; agglutinated with shigella antisera ..... *Shigella*
Non-enteric organisms; optimum temperature below 37 C; water or soil organisms not agglutinated by any shigella antisera ..... *Achromobacter*
76. Motile 77
Non-motile 80
77. Small cocco-bacilli; capsulated; colony mucoid; isolated from the conjunctiva *Noguchia*
Not as above 78
78. Cellulose hydrolyzed ..... *Cellulomonas*
Cellulose is not hydrolyzed 79
79. Yellow colonies ..... *Flavobacterium*
Chromobacterium (T & W.)
Non-pigmented colonies ..... *Achromobacter*
80. Small ovoid cocco-bacilli usually showing bipolar staining; cause hemorrhagic septicemia in animals ..... *Pasteurella*
Not as above 81
81. Colonies adherent to the agar; resemble sulfur granules but are not yellow; organisms may show bipolar staining; strict parasites causing actinobacillosis in man and animals ..... *Actinobacillus*
Not as above 82
82. Cellulose hydrolyzed ..... *Cellulomonas*
Cellulose not hydrolyzed 83
83. Yellow colonies ..... *Flavobacterium*
Chromobacterium (T & W.)
Colonies not pigmented ..... *Achromobacter*
84. Non-motile at 37 C or at 22 C 85
Motile at one of these temperatures 108
85. Grows on MacConkey's agar in three days 86
No growth on MacConkey's agar in three days 95
86. Acid or acid and gas from glucose 87
No acid or gas from glucose 93
87. Acid and gas from glucose 88
Acid only from glucose 89
88. Organisms capsulated; colonies mucoid; organisms pathogenic causing throat infections in man and animals ..... *Klebsiella*
Colonies not mucoid; organisms not capsulated; agglutinated by salmonella group D "O" antiserum ..... *Salmonella*

89. Small ovoid cocco-bacilli; may show bipolar staining; colonies on agar very small, glistening and colorless; organisms cause plague in man and animals . *Pasteurella*
Not as above. . . . 90
90. Colonies pale grey; translucent. . . . 91
Colonies with a yellow pigment. . . . 92
91. Organisms agglutinated by shigella antisera; enteric pathogen. . . . *Shigella*
Not as above. . . . *Achromobacter*
92. Organisms attack alkylamines . . . *Protaminobacter*
Not as above. . . . *Flavobacterium*
Chromobacterium (T & W.)
93. Colonies with a yellow pigment. . . . *Flavobacterium*
Colonies not pigmented . . . 94
94. Litmus milk strongly alkaline; no carbohydrates fermented. . . . *Alcaligenes*
treated in the Miscellaneous groups (T & W.)
Litmus milk acid or unchanged . . . *Achromobacter*
95. Acid produced from glucose. . . . 96
Glucose not fermented. . . . 100
96. Colonies on blood agar very small, transparent, dew-drop or bisected pear; no growth on meat infusion agar; organisms usually small cocco-bacilli but may occur as long spiral filaments on first isolation; inhabitants of the respiratory tract but may be found in large numbers in the cerebrospinal fluid in cases of meningitis in children *Haemophilus*
Not as above; will grow on meat infusion agar. . . . 97
97. Slender rods; 1.0 to 3.0 μ long often arranged in palisades; pleomorphic; will grow on meat infusion agar in 48 hours producing a smooth entire, glistening butyrous colony about 1 mm in diameter; bipolar staining common; café au lait on potato; Strauss reaction in guinea pigs; strict parasites causing glanders especially in man and horses. . . . *Malleomyces*
Small ovoid cocco-bacilli; 0.7 to 2.0 μ long; arranged singly, in pairs and in small bundles; may be pleomorphic and show bipolar staining; will grow on meat infusion agar in 24 hours at 37 C producing a small entire smooth glistening colony which may extend from 1 to 6 mm in diameter in 5 days incubation; no growth on potato; parasites producing hemorrhagic septicemia in animals and birds . *Pasteurella*
Not as above. . . . 98
98. Organisms attacking alkylamines . . . *Protaminobacter*
Not as above. . . . 99
99. Colonies yellow. . . . *Flavobacterium*
Chromobacterium (T & W.)
Not as above. . . . *Achromobacter*
100. Litmus milk acid or alkaline. . . . 104
Litmus milk unchanged. . . . 101
101. Yellow colonies. . . . *Flavobacterium*
Chromobacterium (T & W.)
Colonies not pigmented. . . . 102
102. Colonies 1 to 2 mm in diameter in 24 hours; pale grey, translucent; growth on meat infusion agar good; normal inhabitants of water; saprophytic. . . . *Achromobacter*
Colonies on meat infusion or blood agar 0.5 to 1.0 mm in diameter; often no growth on meat infusion agar on first isolation; cocco-bacilli; parasitic on man or animals. 103
103. Organisms causing subacute infectious conjunctivitis or angular conjunctivitis; occur predominantly as diplobacilli; Loeffler's blood serum slowly liquefied
Moraxella (6th ed.)
Haemophilus (5th ed.)
Isolated from the respiratory tract, meningeal fluid or eye exudates; small cocco-bacilli which require blood or ascitic fluid or other body fluids and sometimes special growth

factors for primary isolation after which some species can be trained to grow on meat infusion media; organisms associated with influenza, whooping cough or meningitis; inspissated serum is not liquefied ..... *Haemophilus*

Organisms associated with undulant fever in man; septicemic infections and abortion in animals; growth good on a liver extract agar; increased CO_2 tension often necessary for isolation ..... *Brucella*

104. Organisms cause acute ophthalmia (pink eye) in cattle; small gram negative rods predominantly arranged in diplobacilli; enriched media necessary for growth; Loeffler's serum liquefied ..... *Moraxella*

Not as above 105

105. Good growth on ordinary meat infusion agar; yellow pigment produced which is insoluble in water ..... *Flavobacterium*
Chromobacterium (T & W.)

Not as above; no yellow pigment 106

106. Litmus milk alkaline 107

Litmus milk acid ..... *Achromobacter*

107. Organisms associated with undulant fever in man and septicemic infections with or without abortion in animals ..... *Brucella*

Not as above ..... *Alcaligenes*

108. Acid and gas produced from glucose 109

Acid only from glucose 110

Glucose not fermented 114

109. Organisms produce a swarming growth on moist agar; gelatin is liquefied or indole is produced, or both ..... *Proteus*

Discrete colonies on moist agar; gelatin is not liquefied and indole is not produced; sucrose and salicin are not fermented; enteric pathogens of man, animals and birds
Salmonella

110. Colonies mucoid; organisms capsulated; isolated from the eye (Rhesus monkeys); parasitic ..... *Noguchia*

Not as above 111

111. Small translucent greyish yellow colonies; mucoid; small slender rods in smooth colonies; ovoid rods with bipolar staining in rough colonies; motile at 37 C; organisms cause melioidosis in man and animals ..... *Malleomyces*
Pfeifferella (T & W.)

Small umbonate granular colonies; translucent with a dull finely granular "beaten copper" surface; entire; butyrous; organisms cause pseudotuberculosis in rodents
Pasteurella

Not as above 112

112. Yellow pigmented colonies ..... *Flavobacterium*
Chromobacterium (T & W.)

Not as above 113

113. Pathogenic; organism responsible for typhoid fever in man; agglutinated by Salmonella group D antiserum ..... *Eberthella* (5th ed.)
Salmonella (6th ed.; T & W.)

Non-pathogenic; not agglutinated as above ..... *Achromobacter*

114. Yellow pigmented colonies ..... *Flavobacterium*
Chromobacterium (T & W.)

Not as above 115

115. Litmus milk rendered strongly alkaline 116

Litmus milk rendered acid or unchanged 117

116. Organisms causing broncho-pneumonia in rodents and sometimes associated with canine distemper ..... *Brucella*

Other than above ..... *Alcaligenes*
(including *Agrobacterium radiobacter*)

117. Small circular translucent colonies; mucoid; organisms causing conjunctival folliculosis in rabbits ..... *Noguchia*
Not as above ..... *Achromobacter*
118. Cocci 119
Rods and cocco-bacilli 120
119. Kidney shaped diplococci ..... *Neisseria*
Cocci in clusters; average diameter 0.3 μ ..... *Veillonella*
120. Minute rod-shaped organisms which grow only in media containing sterile fresh tissue or ascitic fluid; found in the upper respiratory tract of man ..... *Dialister*
Not as above 121
121. Sporing rods ..... *Clostridium*
Non-sporing rods 122
122. Plant pathogens 123
Not plant pathogens 124
123. Peritrichiate flagella ..... *Erwinia*
Bacterium (T & W.)
Polar flagella ..... *Phytomonas* (5th ed.)
Pseudomonas (6th ed.)
124. Long tapered cells, 3 to 16 μ with distinct granules; grow poorly on ordinary meat infusion agar ..... *Fusobacterium*
Never more than 3 μ long; rounded ends; pleomorphic ..... *Bacteroides*

SUMMARY

A mechanical key for the identification of bacteria in the Suborder *Eubacterineae* and the Order *Actinomycetales* is submitted. Attention is drawn to the necessity for precise definitions for species and genera.

The key submitted in this paper was originally prepared as an alternative to the keys provided in the fifth edition of Bergey's Manual of Determinative Bacteriology for placing bacteria into their respective genera. It has since been amended to cover the sixth edition of the Manual and is being submitted in the hope that it will be generally tested and criticized and that its deficiencies will be rectified by suggestions from workers experienced in particular fields.

Directions for the procedure adopted in describing bacteria are available in the majority of textbooks but there are no directions for the application of the information to the identification of the organisms. The only attempt to supply this guidance which covers all types of bacteria and related microörganisms is to be found in Bergey's Manual of Determinative Bacteriology. The attempt has not been very successful. The fault in the Manual keys lies in the attempt to combine a system of classification with a system for identification. An alternative method, which effectively separates the two systems and should benefit both, is suggested in this paper. The key proposed will be capable of marked improvement when bacteriologists provide more adequate descriptions of many listed species and genera but in the meantime it should serve a useful purpose.

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IMMUNOCHEMICAL STUDIES ON BLOOD GROUP SUBSTANCES[1]

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Ever since the discovery of the four major human blood groups by Landsteiner (cf., 35, 37), interest in the chemical nature of the antigens which furnish the basis for this classification has been continual. It was at first believed that there were but two substances, the A and B substances, but it is now clearly established that blood group O is determined not by the absence of the A and B substances but by the presence in the cell of a characteristic blood group substance, called O substance (cf., 9, 53). Studies of earlier investigators, Landsteiner (35, 37), Schiff (49), Witebsky (54–55), Meyer, Smyth and Palmer (41), Freudenberg, *et al.* (13), Goebel (14) and others, have established that these antigens or, as they are usually termed, blood group substances, are widely distributed throughout the animal kingdom, and water-soluble products possessing blood group A, B, or O activity, alone or in combinations, have been obtained from tissues or secretions of various animal species (cf., 9, 37, 53). For instance, hog gastric mucosa (39, 41), commercial preparations of peptones (49), swine pepsin (38), and the fourth stomach of the cow (abomasus) (24) have been shown to contain blood group A substance, and horse saliva and stomach to contain blood group A and B substances (36, 34, 57). Tissue fluids and secretions, such as serum, saliva (9, 30), gastric juice (55), urine (13), etc. from 80 per cent of humans of a particular blood group contain the corresponding blood group substances in soluble form; individuals are classified as secretors or non-secretors depending upon whether their secretions (usually saliva) contain blood group substance (cf., 9, 37, 48, 53).

The activity of blood group substances in solution is usually measured by their capacity to combine with homologous antibody and to prevent the antibody from agglutinating erythrocytes. Assays are carried out by the usual serological dilution methods and the activity of various preparations may be compared by measuring the minimum quantity of each capable of neutralizing an arbitrary number of hemagglutinating units of antibody (cf., 33, 53). With this hemagglutination inhibition technic as a guide, a number of investigators, notably Schiff (49), Goebel (14), Witebsky *et al.* (55), Landsteiner and Harte (39), Morgan (44–46) undertook to purify these materials from a variety of sources and obtained products which were predominantly polysaccharide in nature. Landsteiner and Harte (39), however, demonstrated that even their purest preparations contained amino acids and these observations were confirmed by other investigators (44, 7).

From the standpoint of the immunochemist, however, the assay of blood

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group activity by the hemagglutination inhibition technic was relatively unsatisfactory, because of the large errors involved and the consequent difficulty of comparing the activity of various preparations with one another and of correlating activity and other chemical properties. If a precipitin reaction between A substance and homologous anti-A formed in humans could be demonstrated, it would immediately be possible to apply the powerful technics of quantitative immunochemistry (cf., 16, 25, 26, 33, 51) to the assay of these substances and to obtain estimates of their purity.

Three important developments took place which prepared the way for the demonstration of such a precipitin reaction. The first of these was the development of a micro modification of the quantitative precipitin technic by Heidelberger and MacPherson (21). This method involved the use of relatively large volumes of serum (as much as 4 ml per determination) and allowed intervals of 7 to 8 days in the refrigerator for precipitation to take place. In this manner quantities of antibody nitrogen as low as 10μg could be measured with a precision of ± 2 μg N. The method proved especially valuable in the assay of the antibody response in humans during convalescence from pneumonia (17) or following immunization with the type specific pneumococcal polysaccharides (20).

The second was the introduction of the Morgan and King phenol method (44) for the purification of the blood group A substance from hog gastric mucin. This procedure involves extraction of the mucin with 90 per cent phenol, and precipitation of the blood group A substance from the phenol extract by the addition of alcohol to a concentration of 10 per cent by volume. The third development was the finding by Witebsky, Klendshoj and McNeil (56) that blood group A and B substances were antigenic in humans who lacked these factors and that the anti-A and anti-B titers in such individuals could be readily increased by the injection of small quantities of such substances.

Our laboratory was interested in the blood group substances and it appeared desirable to investigate the possibility of obtaining a precipitin reaction between A substance and anti-A by these newer microtechnics. It was soon apparent that specific precipitation between A substance and anti-A and between B substance and anti-B occurred but that the quantities involved were not detectable by the usual methods (32). For instance, before immunization the serum of one individual of group O had an anti-A titer of about 8 to 16 and contained about 2 to 3 μg anti-A nitrogen per ml; after immunization the anti-A titer had increased to 512 and the precipitin content to 54 μg anti-A N per ml. The rise in titer and in precipitin content of the sera ran parallel within experimental error. Injection of A substance into individuals of groups A or AB, or of B substance into individuals of groups B or AB did not produce any hemaglutinin and gave rise to negligible quantities of precipitin. The precipitin reaction of A substance from gastric mucin with anti-A produced in humans immunized with A substance was studied quantitatively and found to be of the usual type. Maximum precipitation of anti-A occurred when a slight excess of A substance was present in the supernatant (32).

The quantitative precipitin method provided a more precise assay of the relative quantities of blood group substance in various preparations. This could

readily be accomplished by adding known quantities of the samples containing A substance to be tested to a measured volume of anti-A and determining the quantity of antibody nitrogen precipitated. By interpolation of this value on a calibration curve, relating the quantity of antibody nitrogen precipitated by varying known amounts of a blood group A preparation arbitrarily chosen as a standard, the relative capacity to precipitate anti-A of the preparation to be tested in terms of the standard could be determined (cf., 33). By this technic a variety of blood group A substances were compared and the stability of the A substance at various pH levels was investigated (29).

To obtain additional information about the purity of the blood group substances it was decided to obtain materials from individual hog stomach linings instead of from a random pool of hog stomachs such as represented by gastric mucin. Witebsky (54) had previously noted that only two of five hog stomachs showed blood group A activity and it seemed possible, by selection of hog stom-

TABLE 1*

Properties of Blood Group A and O Substances from Individual Hog Stomach Linings

	A	O
No. of stomachs	7	3
Nitrogen %	5.9–6.6	5.7–6.1
Reducing sugar %	55–61	56–59
Glucosamine %	32–34	32–34
Acetyl %	9.3–11.3	9.4–9.9
Rel. viscosity	1.39–1.71	1.47–1.63
Electrophoretic mobility pH 7.4 cm^2/volt-sec	-1.4×10^{-5}	-1.3×10^{-5}
Constituents isolated	L-fucose D-galactose D-glucosamine	L-fucose D-galactose D-glucosamine

* From (7, 8).

achs showing A activity, that products of higher purity might be obtained. Accordingly the stomach linings from 10 individual hogs were digested with pepsin or allowed to autolyze, the digests precipitated with 5 volumes of ethanol and the precipitates dried and purified (7) by the Morgan and King phenol method (44). Although only seven of the 10 stomachs had shown A activity, products were obtained in similar yield from all ten stomachs which were identical in nitrogen, glucosamine, reducing sugar, acetyl and in electrophoretic mobility. The range of analytical properties found (7, 8) for the preparations showing A activity and those which did not are given in table 1. Bray, Henry and Stacey (10) had established the presence of L-fucose in preparations of A substance obtained from pools of hog stomachs and it was possible to isolate derivatives of L-fucose, D-glucosamine and D-galactose from the products showing A activity and from those which did not show A activity (8).

The products which had no A activity were shown to possess O activity as evidenced by their ability to inhibit hemagglutination of O cells by cattle anti-O (8). Morgan and Waddell (46) had noted the presence of O activity in A prepara-

tions from hog gastric mucin and Aminoff, Morgan and Watkins (3) reported that the alcohol precipitates from autolysates of hog stomachs contained either A or O activity. Purified products obtained from individual hog stomach linings were shown to possess either A activity alone or O activity alone or in some instances products, presumably from heterozygous hogs, exhibited both A and O activity (8). Representative data are shown in table 2. These findings have recently been confirmed (10a).

The capacity of the A preparations from individual hog stomach linings to precipitate anti-A produced by immunization with hog A was assayed. Six of

TABLE 2*

Assay of Blood Group Substances for O Activity by Inhibition of Hemagglutination of Cattle Anti-O

MICROGRAMS SUBSTANCE ADDED	20	5	2	0.5	0.2	PROPORTION OF GLUCOSAMINE PRECIPITABLE BY ANTI-A
Samples showing A activity						
Hog 1	+±	+++	+++	+++	+++	83
3	+++	+++	+++	+++	+++	90
4	+++	+++	+++	+++	+++	90
5	+++	+++	+++	+++	+++	84
Samples showing both A and O activity						
9	−	+	++±	+++	+++	70
32	−	−	−	+	++±	10
15	−	±	+	++	+++	(73)
Samples showing O activity						
2	−	−	−	−	+++	(0)
6	−	−	−	+±	+++	(0)
7	−	−	−	+++	+++	(0)

* From (8).

the seven A preparations from individual hog stomachs were identical in their precipitating power for anti-A (7). The seventh was somewhat less active and was found to possess some O activity (7, 8). A sample of blood group substance from hog gastric mucin, containing both A and O substance was found to have only about 58 per cent of the precipitating power for anti-A of the materials from individual A stomachs (7).

The purified preparations showing only blood group O activity gave negligible quantities of precipitate with anti-A. Immunization of humans of all four blood groups with O substance did not give rise to any precipitins for the A or O substances. These observations provide further evidence that the precipitin formed on immunization of humans of blood group O and B with hog A substance is indeed anti-A (7, 8, 32).

A major question to be decided was whether the products obtained from the A and O stomachs by the methods used, actually were the blood group A and O substances. No evidence on this point had previously been adduced and a likely assumption might be that the bulk of the products obtained consisted of impurity of the composition indicated by analysis and that this impurity was contaminated by a small amount of A substance if obtained from an A hog or by a small amount of O substance if obtained from an O hog.

It was possible to arrive at a definitive exclusion of this latter possibility by quantitative immunochemical methods. Studies on the quantitative precipitin reactions of well defined single antigens and their homologous antibodies have established that all of the antigen is precipitated by an excess of antibody. Heidelberger and Kendall (19) showed that practically all of the color of a red azo dye protein antigen was precipitated by an excess of antibody. Stokinger and Heidelberger (50) demonstrated that 96 to 101 per cent of the iodine in thyroglobulin was precipitated by an excess of antibody. Similarly Hooker and Boyd (23) found all of the copper of hemocyanin to be present in specific precipitates formed with excess antibody.

From these considerations, an estimate of the absolute purity of the blood group A preparation could be obtained by analyzing washed specific precipitates formed in the region of antibody excess for some characteristic constituent present in the preparation added. The most suitable constituent for this purpose was glucosamine since it comprised about one-third of the weight of the blood group preparations. If only a negligible proportion of the weight of the samples was blood group substance then the specific precipitates formed with the A preparations and anti-A should contain little or no glucosamine. Conversely, if the A preparation were pure A substance, essentially all of the added glucosamine, within experimental error should be found in the precipitate (cf., 7).

Representative results for a sample of blood group A substance from an individual hog stomach and from a specimen from hog gastric mucin are shown in table 3. The specific precipitate formed by addition of excess anti-A to 90 μg of Hog 1 substance (29 μg of glucosamine) contained 33 μg of glucosamine or 114 per cent of the added glucosamine, while that obtained under similar conditions from 120 μg of sample 1A (36 μg of glucosamine) contained 28 μg of glucosamine or but 78 per cent of the quantity of glucosamine added. Two correction factors must be applied to these values. One of these is a correction for the solubility of the A-anti-A specific precipitate. The quantities of antibody N precipitated by equivalent amounts of A substance were corrected to zero volume by adding 1.6 μg N dissolved per ml for the total volume in which the analysis was carried out. A solubility correction factor was obtained by dividing the antibody N corrected to zero volume by the uncorrected value and the glucosamine found in the specific precipitate was corrected for solubility by multiplying by this solubility correction factor. The correction for solubility increased the amounts of glucosamine in the specific precipitates (table 3) by 7 to 8 per cent.

The second correction factor is one for the glucosamine content of the antibody. This was estimated by determining the ratio of glucosamine to nitrogen in

normal human gamma globulin and multiplying the quantity of antibody N precipitated, corrected for solubility, by this ratio 0.07. Subtracting the glucosamine content of the antibody from the glucosamine content of the specific precipitate after correction for solubility gave 24 μg of the 29 μg of hog 1 glucosamine precipitated by anti-A and but 20 μg of the 36 μg of the hog mucin sample 1A precipitated by anti-A. These values represent 83 and 56 per cent of the added glucosamine (table 3). The values for the six individual hog stomachs showing only A activity varied from 75 to 90 per cent with an average of 84 per cent of the glucosamine precipitable by anti-A (7), and repeated determinations on sample 1A gave an average of 54 per cent. The ratio of 54/84 or 64 per cent for the proportion of the glucosamine of sample 1A to that of the individual hog

TABLE 3*

Glucosamine Analyses on A-anti-A Specific Precipitates

	HOG 1	1A
Micrograms substance added	90	120
Micrograms glucosamine added	29	36
ml serum used	3	3
ml total volume	6	6
Micrograms glucosamine in ppt	33	28
Percent glucosamine recovered	114	78
Micrograms antibody N in ppt. found	144	126
Micrograms antibody N in ppt. corrected for solubility**	154	138
Solubility correction factor	1.07	1.08
Micrograms glucosamine in ppt. corrected for solubility	35	30
Glucosamine due to antibody (N × 0.07†)	11	10
Micrograms glucosamine due to A substance	24	20
Percent of added glucosamine in specific precipitate	83	56

* From (7).

** Solubility of A-anti-A precipitates 1.6 μ g N/ml.

† Glucosamine content of human gamma globulin.

stomachs precipitable by anti-A is in good agreement with the value of 58 per cent for their relative capacities to precipitate anti-A. Only 70 per cent of the glucosamine of blood group substance from a seventh hog stomach (hog 9, table 2) which contained some O substance was precipitable by anti-A. In another instance, hog 32, which was predominantly O substance (table 2), only 10 per cent of the glucosamine was precipitated by anti-A. The values are subject to the uncertainty that the ratio of the glucosamine to nitrogen contents of anti-A might differ slightly from that of human gamma globulin (7, 8).

These findings, however, clearly exclude the possibility that only a small proportion by weight of the purified blood group A preparations is blood group A substance. Accepting the average of 84 per cent of the glucosamine of the preparations as precipitable by anti-A, and since the samples contain 33 per cent of glucosamine, at least 28 per cent (0.84 × 33) of the weight of the blood group A substance from individual hog stomachs showing A activity has been shown to

be involved in the reaction with anti-A (7). These conclusions would not be affected by the report of Aminoff and Morgan (1) based on paper chromatography, that chondrosamine is present in the blood group A substance in addition to glucosamine. The method of analysis measures total hexosamine and the values referred to as glucosamine may actually be glucosamine plus chondrosamine, each of which give equal color values per unit weight in the glucosamine analysis.

Human blood group A substances have been prepared from human saliva by a process of peptic digestion, alcohol precipitation, and phenol purification (30). The analytical properties of the human blood group A substances are generally similar. Unlike the hog substances, however, human blood group A substances occur in two fractions a phenol-insoluble fraction, and a portion soluble in phenol and precipitable from phenol at a concentration of 10 per cent ethanol. Analyses of specific precipitates of blood group A substances from human saliva, have shown that with the best preparations essentially all of the glucosamine was precipitated by anti-hog A (30).

Additional information on the blood group substances was obtained by a study of the cross reaction of the substances with Type XIV antipneumococcus serum. The Type XIV specific polysaccharide contains two of the sugar constituents present in the blood group substances, namely N-acetyl D-glucosamine and D-galactose; and Type XIV serum was found to agglutinate A, B, AB, and O erythrocytes (5, 12, 52; cf., 33) and the blood group O substance has been shown to inhibit hemagglutination of O erythrocytes by Type XIV antibody (57). In addition a sample of blood group A substance from pepsin had been shown to precipitate with Type XIV antibody (5, 15). The purified blood group A and O substances from individual hog stomachs, as well as purified human A, B, and O substances were also found to precipitate with Type XIV antibody; these observations providing a chemical basis for the agglutination of erythrocytes of all four blood groups by type XIV horse antibody (31).

A very surprising finding in the cross-reaction of the blood group substances with Type XIV antibody was that individual preparations of blood group A substances, which were identical in their reactivity with anti-A varied widely in their capacity to precipitate Type XIV antibody. For instance, 500 μg of blood group A substance from hog 10 precipitated only 19 μg N from 0.5 ml Type XIV serum while equal quantities of similar products from hogs 16, 3, and 8 precipitated 39, 72 and 72 μg N respectively. A similar range of variation in precipitating power for Type XIV antibody was observed with hog blood group O substances as well as with blood group A and O substances from human saliva (31).

In an attempt to understand the basis for this difference in cross-reactivity of preparations with identical blood group A activity, the pH stability range of the blood group A substance was investigated with emphasis on the effects on cross reactivity (27). Earlier studies (29) had shown that the capacity of the blood group A substance to precipitate anti-A was unaffected by heating at 100 C for 2 hours over a pH range of from about 3 to 7.5; loss of activity occurred on exposure to 100 C at pH 1 and at pH 9.0 or above. With respect to

cross reactivity with Type XIV antibody, however, it was found that heating at 100 C for 1 to 2 hours at pH of 1.5 to 1.8 resulted in complete loss of blood group A activity but caused a striking increase in the capacity of the material to precipitate with Type XIV antibody. For instance 500 μg of hog 10 originally precipitated 19 μg N from 0.5 ml Type XIV serum but after 2 hours at 100 C at a pH of 1.59, the same quantity of substance precipitated 64 μg N from 0.5 ml serum; with hog 16, the change on heating was from 39 to 70 μg N. This effect of heating at acid pH on cross reactivity was found with hog O substance as well as with human A and B substances and appears to be characteristic of blood group substances (27).

TABLE 4*

Dialyzable Constituents Liberated by Mild Hydrolysis of Hog A and O Substances

	HOG 15 (A)	HOG 29 (O)
	mg	*mg*
Amount substance used	260	265
Conditions of hydrolysis	2 hours, pH 1.5	1 hour, pH 1.8
	Found in dialysate	
Reducing sugar hydrolyzed	57	30.6
unhydrolyzed	31	15.3
Glucosamine hydrolyzed	23	7.4
unhydrolyzed	4.3	1.2
Fucose	17.1	17.9
Nitrogen	3.2	2.2
Non-glucosamine N	1.4	1.6

* From (27).

To ascertain some of the chemical changes which took place on heating at pH 1.5 to 1.8 and which might be responsible for this increase in cross reactivity, 260 mg of blood group A substance (hog 15) was heated at 100 C at pH 1.5 for 2 hours. The solution was then dialyzed, the dialysate concentrated in vacuo under CO_2 and its composition determined by analysis and by paper chromatography. The results (27) are given in table 4. The dialysate contained 17.1 mg of free fucose, about 80 per cent of the quantity initially present in the 260 mg of A substance (hog 15). In addition it contained partially depolymerized glucosamine-galactose residues as evidenced by the increase in glucosamine and reducing sugar on further hydrolysis of the dialysate. It also contained 1.5 mg of non-glucosamine N. Similar findings were obtained with a blood group O substance (hog 29, table 4), except that about sixty per cent of the fucose was split off under the somewhat milder conditions of hydrolysis. Isolation of the non-dialyzable portion of the blood group substances after the mild hydrolytic procedure yielded products with increased cross reactivity with Type XIV anti-

serum and with much lower methylpentose contents than the original blood group substances, the other analytical properties were not significantly altered except that the optical rotation became more positive (27).

These chemical and immunochemical findings permit certain inferences to be drawn as to a portion of the structural pattern of the blood group substance. The Type XIV specific carbohydrate (SXIV) and the hog blood group A and O substances have been shown to consist of N-acetyl-glucosamine and galactose residues. By virtue of the cross-reaction of the blood group substances with Type XIV antiserum, it would seem reasonable that both the SXIV and the blood group substances possess in the basic chain, which need not necessarily be a straight chain, a series of N-acetylglucosamine-galactose residues; the sequence in the various substances not necessarily being identical, but giving rise to patterns with certain similarities of structure at various points on the chain, much as in the case of the structures proposed for SIII and SVIII to explain their cross-reactivity with their heterologous antipneumococcal horse sera (18). The blood group substances, unlike SXIV, contain fucose residues and by methylation studies, Bray, Henry and Stacey (10) have shown these to be present as end groups. If these fucose end groups were attached to the main N-acetyl-glucosamine-galactose chain at various points, they would project outward and might very well prevent the cross-reacting groupings from combining with antibody. Removal of these fucose residues might either provide additional cross-reacting sites on the main chain or might make possible a closer approach of the antibody molecule to the reactive groupings on the main chain and thereby increase the extent of the cross-reaction as has been observed (27). The fact that the fucose may be easily removed without effecting appreciable hydrolysis of the main galactose-glucosamine chain is further evidence that the fucose does, indeed, occur as end groups and is not an integral part of the main polysaccharide chain.

These data, however, provided no explanation for the variation in the capacity of individual blood group substances to precipitate Type XIV antibody. The development of a specific color reaction for methylpentoses by Dische and Shettles (11) made it possible to determine the methylpentose contents of the various blood group samples (4). The fucose contents of the individual hog blood group A or O samples varied from about 6 to 13 per cent with an average of 9 per cent. Similar variation was noted among preparations of human blood group substances, but the values were somewhat higher ranging from 9 to 16 per cent with an average of about 13 per cent.

A plot of the total N precipitated from 0.5 ml Type XIV antipneumococcal horse serum by 500 μg of the various hog blood group A and O substances is given in fig. 1. It is apparent that there is an inverse relationship between fucose content and cross reactivity. This is in accord with the concept advanced above that the fucose residues project as end groups from a chain of N-acetylhexosamine-galactose residues and that these fucose residues reduce the cross reactivity with Type XIV antibody. Fucose values as high as 18 per cent with complete

absence of cross reactivity for human blood group substances are probably somewhat unusual in view of the agglutination *in vivo* and *in vitro* of human A, B, AB, and O erythrocytes by Type XIV antipneumococcal horse serum (12, 52).

No such correlation between the fucose content and cross reactivity was found among the individual preparations of human blood group substances. However, a sample of blood group A substance from human ovarian cyst fluid (45) obtained from Dr. W. T. J. Morgan at the Lister Institute was found to contain 18 per cent fucose (28), the highest value of any sample tested. It did not react with

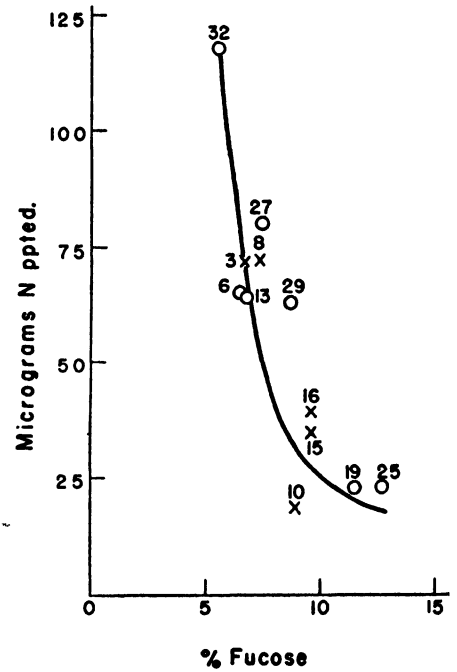

FIG. 1. Relation between fucose content and cross-reactivity of hog A and O substances with Type XIV antipneumococcal horse serum H635. × = hog A substances; O = hog O substances. Ordinate = micrograms N precipitated by 500μg blood group substance from 0.5 ml antiserum. (Ref. 4, Journal of Experimental Medicine.)

Type XIV antiserum until subjected to mild acid hydrolysis, providing further evidence of the role of the fucose in blocking cross reactivity. These observations were confirmed in Dr. Morgan's laboratory (3). This group also found that the capacity of the A substance to inhibit hemolysis of sheep erythrocytes by rabbit antiserum to human A cells was enhanced after removal of fucose by mild acid hydrolysis (cf., Landsteiner and Harte (39)).

The availability of a specific method for the estimation of fucose made it possible to determine the proportion of the fucose of the blood group A substances specifically precipitated by excess anti-A as had been done for the hexosamine of the A substance (28). The data obtained indicated that, within experimental error, essentially all of the fucose of the best hog and human blood

group A substances was specifically precipitable by anti-A. This was independent of the variation in fucose content among the individual preparations.

By analysis of specific precipitates for both hexosamine and fucose, about 34 to 38 per cent of the weight of the hog A substances and as much as 45 per cent of the weight of a human blood group A substance has been shown to be precipitated by anti-A (28). From the cross reaction studies and the demonstration that the hexosamine is present as N-acetyl hexosamine (cf., 43), it is reasonable to assume that both the galactose and the acetyl could also be precipitated by anti-A. This would raise the weight of material associated with A activity to about 70 to 80 per cent and leave only the role of the amino acids to be established. Small amounts of impurities may still be present in the preparations; Holzman and Niemann (22) have attributed variations in the absorption at 2600 Å among different preparations of blood group A substance to an impurity but no data on the amounts present are available.

While the chemical and immunochemical studies to date have led to the preparation of blood group substances from several species in fairly pure form, have produced methods for their characterization and the estimation of their absolute purity on a weight basis, and have also provided some slight inkling as to their structure, the major problems await further study. Nothing is known of the structural relationships determining blood group A, B, or O activity and specificity, the most important and difficult phase of the problem. Recent studies are providing more and more evidence that the blood group A substances of different species are not identical but show species differences and cross-reactions essentially similar to those found for the serum and tissue proteins. Species differences among blood group B (6) and O (47) substances have also been reported. The intensive immunochemical investigation of such species differences appears to be a promising approach to study further the fundamental chemical basis of blood group activity.

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NOMENCLATURE AND CLASSIFICATION OF INSECT VIRUSES[1]

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Although there were earlier attempts to name and classify viruses, the subject gained new and critical attention when the sixth edition of *Bergey's Manual of Determinative Bacteriology* for the first time included a 160-page supplement titled "Order Virales, The Filterable Viruses." This supplement was prepared by Francis O. Holmes, and was an extension of an earlier preliminary presentation published by him in 1939. It included animal viruses, plant viruses, and bacterial viruses (bacteriophages). As organized by Holmes, the order Virales consists of 13 families, 32 genera, and 248 species.

One's philosophy as to whether or not the time is yet ripe to name and to attempt to classify the viruses may fall within a range from violent objection to at least sympathetic acceptance of the idea. There are those who feel that since we have little or no information as to how viruses multiply, these agents should not be treated in the same manner as are the bacteria which, in general, divide by fission. The fact that certain of the plant viruses have been isolated in the form of crystalline proteins also causes some to question the appropriateness of applying a binomial nomenclature to viruses. A more realistic approach, however, would seem to be the acceptance of the fact that there is a distinct need for some kind of generally accepted nomenclature for these agents as is evidenced by several past attempts at nomenclatures using numbers, letters, and other designations. Furthermore, the presentation made by Holmes in the Bergey *Manual* constitutes a formal and scientifically valid proposal which should be dealt with in a scholarly manner. On a general ideological basis, the writer is inclined to accept the Holmes classification as a provisionally satisfactory starting point. Furthermore, even though in this paper certain constructive criticism will be offered concerning points of pertinent detail regarding Holmes' treatment of insect viruses, the writer wishes to make clear his appreciation of the great amount of thought and work that this author has put into a commendable pioneer presentation.

So far as the appropriateness of applying the binomial system of nomenclature to the viruses is concerned, with certain possible exceptions (*e.g.*, the advisability of recognizing the viruses as a new kingdom), the writer finds himself in rather general agreement with the point of view expressed by R. E. Buchanan in a talk at the "Bergey's Manual Dinner" in Cincinnati, May 17, 1949, and since then circulated in mimeographed form. In his discussion, Buchanan acknowledges several inadequacies of Holmes' systematics but believes that none of them is fundamental and that there appears to be no good reason why the binomial system should be avoided. The writer also recognizes the statement in the In-

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ternational Bacteriological Code of Nomenclature: "Bacteriological nomenclature considers bacteria, related organisms, and the viruses."

The present paper is concerned with the nomenclature and classification of only those viruses which infect and cause diseases in insects; it does not deal with those viruses, of other animals and of plants, which are transmitted from host to host by insects. Furthermore, it is to be recognized that some of the generalizations made with regard to the insect viruses may not apply to plant and animal viruses in general.

HISTORICAL ASPECTS IN BRIEF

As it was with the virus diseases of plants and animals generally, so it was with the virus diseases of insects—the first ones observed were attributed to other causes. In addition to miasmic causes, early investigators assigned etiological roles to certain protozoa, bacteria, and fungi.

Probably the first recognized disease of insects to be caused by what is now known to be a virus was jaundice disease of the silkworm (*Bombyx mori* (Linn.)). Records as far back as the early 16th century refer to what is very probably this polyhedrosis. It was not until the early part of the 20th century that the virus nature of this disease was definitely acknowledged. Not only was silkworm jaundice the first known virus disease of insects to have been discovered, but even today probably more is known concerning it than any other of the more than a hundred reported virus diseases of insects. Similarly, the agent of silkworm jaundice has served as the center for the development of a nomenclature for the insect viruses.

Believing the agent of silkworm jaundice to be a protozoan, Bolle (1894) gave it the name *Microsporidium polyedricum*. It was Bolle's conception that the polyhedral inclusion bodies were the spores of the protozoan which multiplied in a manner similar to that of coccidia. Von Prowazek (1907) at first also believed that a protozoan was responsible for the disease, but that the polyhedra themselves were simply by-products of the infectious process. He gave to the agent the name *Chlamydozoon bombycis*. Prell (1918) considered the parasite to be located within the polyhedron and that the granules sometimes seen in the inclusion represented the nuclei of the causative organism for which he proposed the new generic name *Crystalloplasma* with the agent of silkworm jaundice, *Crystalloplasma polyedricum* (Bolle), as the type species. Later, Prell (1926) gave the name *Crystalloplasma monachae* to the agent of *Wipfelkrankheit*, a polyhedrosis of the nun-moth caterpillar (*Lymantria monacha* Linn.).

By the time of Prell's contributions, the idea that jaundice disease of the silkworm, as well as similar diseases of other insects, might be caused by a filterable virus was being advanced by several workers, notably Acqua (1918–19) and Paillot (1924 *a*, *b*, 1925, 1926 *a*, *b*, 1930). Paillot identified the virus with certain minute, ultramicroscopic granules which could be demonstrated with the aid of a darkfield microscope. To these granules, which he considered the true cause of the disease, Paillot gave the name *Borrellina bombycis*. The generic name *Borrellina* honored the name of the French bacteriologist, A. Borrel. This genus

has retained its validity and includes those viruses which cause the formation of polyhedra in the tissues of the diseased host. According to Holmes (1948), Paillot's original spelling of the generic name with two l's is an error, and the name should be spelled *Borrelina*.

Mention should be made here of a lengthy paper by Del Guercio (1929) dealing with the taxonomy of what he believed to be the etiological agents of polyhedral diseases of numerous insects of Italy. Just what Del Guercio actually saw is difficult to say; they were unlike any known microörganism. He believed them to be organisms phylogenetically located between fungi and algae on the one hand and true bacteria on the other. He characterized them as being microscopic, thallus-shaped, arborescent vegetative growths, having a ramified, involved structure growing out of a stroma, and having fruiting bodies of polyhedric form with some species also having small coccus-like reproductive forms. Of 71 species reported, Del Guercio named 66. These species were separated into 12 genera. The type genus was *Entomococcus* of which the type species was *Entomococcus bombycinus*, the supposed cause of silkworm jaundice. The drawings which illustrate the paper indicate the incongruity of the work, and are difficult to interpret from a biological standpoint. As the writer has indicated elsewhere (1949), it seems unnecessary to give serious consideration to the names proposed by Del Guercio since it is quite obvious that whatever were the forms that he saw, they certainly were not viruses, and would appear to have no phylogenetic relation of any kind to the agents which cause typical polyhedroses.

This brings us to a consideration of the nomenclature proposed by Holmes (1948) in the sixth edition of *Bergey's Manual of Determinative Bacteriology*, and an evaluation of the generic categories.

GENERA OF INSECT VIRUSES

The classification of insect viruses as presented by Holmes groups these agents into two genera, *Borrelina* and *Morator* in the family Borrelinaceae, suborder Zoophagineae, order Virales. The genus *Borrelina* is characterized as consisting of "Viruses inducing polyhedral, wilt, and other diseases; hosts, Lepidoptera, so far as known." The genus *Morator* is given the following description: "Only one species at present, inducing the disease known as sacbrood of the honey bee."

The inadequacies of these descriptions are probably apparent to most students of insect viruses, although it should be admitted that the knowledge concerning these viruses was poorly organized even as recently as when Holmes prepared his classification. Nevertheless, enough information was at hand to have been more exacting in the delimitation of genera. Thus, Holmes' statement that members of the genus *Borrelina* are "known only as attacking lepidopterous insects," although true for the species he recognizes, does not take into account the polyhedroses of certain Hymenoptera and Diptera. His assertion that members of the genus *Morator* are "known only as attacking the honey bee, a hymenopterous insect," could perhaps have been broadened to include the virus (*Borrelina flacheriae* Paillot) responsible for initiating the diseases of gattine and true flacherie of the silkworm, a lepidopterous insect, although the uncertainty which

surrounds the nature of this virus could reasonably justify its omission from this genus, at least for the time being. To be more logically questioned is the advisability of including *Borrelina brassicae* Paillot and *Borrelina pieris* Paillot in the genus *Borrelina*, as was done by both Paillot and Holmes. These viruses not only do not give rise to polyhedral inclusions (but rather "granules") in the infected cells of the host, but also produce a distinctly different type of disease from that caused by the socalled polyhedral viruses.

To assist in correcting and clarifying some of these systematic vagaries, the writer has recently proposed (Steinhaus, 1949) that the known insect viruses be considered tentatively as consisting of four principal groups according to the type of inclusion bodies formed when the latter are present. Thus, those viruses causing insect diseases characterized by the presence of polyhedral inclusions in the infected cells of the hosts are placed in the genus *Borrelina* Paillot; those viruses causing insect diseases characterized by the presence of refringent, polymorphic inclusions of very irregular shape and size in the cytoplasm of the infected cells of the host are placed in the genus *Paillotella* Steinhaus; those viruses causing insect diseases characterized by the presence, in large numbers, of very small but microscopically visible granular inclusions in the infected cells (particularly visible in the cytoplasm) of the host, are placed in the genus *Bergoldia* Steinhaus; and those viruses causing insect diseases in which no visible pathological inclusion body of any kind is produced are placed in the genus *Morator* Holmes.

In all probability, the viruses causing diseases in insects are not limited to the four genera just mentioned. Indicative of this is the likelihood that the infection described by Weiser (1948) as occurring in larvae of *Camptochironomus tentans* (Fabr.) may be caused by a virus representative of a fifth genus. Indeed, we should be inclined herewith to recognize such a genus were it not for the fact that the virus itself has not yet been isolated or demonstrated with the electron microscope or other defining physical means. As will be explained shortly, the writer is of the opinion that henceforth and until other criteria are established, no new specific names should be proposed unless the viruses concerned have at least been demonstrated with the electron microscope or by other means which delineate their basic physical and morphological properties. In the present case, the virus not having been demonstrated, it is not possible to designate the required type species for the genus and at the same time adhere to the specifications just mentioned. It may be pointed out, however, that the disease described by Weiser differs from those caused by the viruses of the four named genera primarily by the fact that the relatively large oval inclusion bodies occur in the cytoplasm of the infected cell (the nucleus remaining undamaged and of normal size), and these bodies contain numerous small granules which can be seen in the inclusions even without alkaline treatment.

TYPE SPECIES

Of the four genera into which the insect viruses are grouped, two (*Borrelina* and *Paillotella*) have type species originally named by Paillot as members of the

genus *Borrelina*. In neither of these instances is it certain that this French worker actually saw the virus. In the case of jaundice disease of the silkworm (caused by *Borrelina bombycis*) he observed certain minute granules, invisible by ordinary microscopy but visible with the aid of a darkfield microscope, in the blood and tissues of the diseased insect. Paillot believed these granules to be the causative agent of the disease and it was to them he gave the name *Borrellina bombycis* [*Borrelina*]. The exact relation these granules of Paillot's have to the virus still remains to be determined. Since then the actual virus has been isolated and demonstrated with certainty by means of the electron microscope (Bergold, 1947), and has been shown to be a rod-shaped particle approximately 40 by 288 millimicrons in size. In spite of the uncertainty as to the identity of the granules described by Paillot, it is acknowledged that this worker knew he was concerned with a filterable virus and that the name he gave the granules was intended for the etiologic agent of the disease which he believed to be identical with them. Names used prior to that of *Borrelina bombycis* were not favored with as acceptable an insight as to the true cause of the polyhedrosis concerned (most earlier writers thought they were naming a protozoan) and hence they have been generally ignored. Consequently the name proposed by Paillot has been accepted as the name of the virus. In this we follow Holmes' acceptance of Paillot's nomenclature for those species which he named and recognized as associated with certain of the insect virus diseases. It so happens therefore that the specific epithets proposed by Paillot have become those of the type species of each of the genera *Borrelina* and *Paillotella*. The two species concerned are *Borrelina bombycis* Paillot and *Paillotella pieris* (Paillot) Steinhaus. The exact nature of *Paillotella pieris* is unknown; it is the only species known for the genus, and the disease it causes is the only one of its kind so far reported.

At the time the writer (Steinhaus, 1949) proposed the name *Bergoldia* for the genus of viruses which cause those diseases provisionally known as granuloses, the only species bearing a name was *Bergoldia brassicae* (Paillot) Steinhaus (= *Borrelina brassicae* Paillot). Unfortunately, this species was so inadequately known as compared with certain others of the group that it would have been a very unsatisfactory type species for the genus. Furthermore, the genus could not be adequately characterized from what was known of this species alone. Accordingly, in defining the genus, the writer indicated as type species of the genus the virus responsible for the granulosis (Bergold's *Kapselvirus-Krankheit*) of *Cacoecia murinana* (Hbn.), which is the first one of the group to have been isolated in a free state (Bergold, 1948) and one about which relatively much is known. The species was named *Bergoldia calypta*.

The type species of the remaining genus, *Morator*, is *Morator aetatulae* Holmes, the cause of sacbrood in honeybees.

CLASSIFICATION OF INSECT VIRUSES

In recent years considerable progress has been realized in an understanding of the basic nature of the insect viruses. In order to bring the information pertaining to the classification and nomenclature of this group up to parallel these

new advances, the arrangement which follows is presented. In making this presentation, however, the writer is under no illusions as to its infallibility. Indeed, it is offered strictly in a tentative and provisional sense, and with the hope that it will serve as a satisfactory starting point for more profound treatment by others.

With regard to the naming of new species of insect viruses, the writer wishes to make the proposal that henceforth new species be named only after the virus itself has been demonstrated as a morphologically distinct entity by accepted physical or visual means. It is admittedly difficult to decide on the criteria that should be used in naming species of viruses. It would seem, however, that there is something fundamentally wrong in describing as a new species an agent which has not even been seen or whose gross morphological aspects have not been determined. At any rate, so far as the insect viruses are concerned, the electron microscope has been able to reveal most species which have been diligently sought. In keeping with this belief, in the present paper the writer is assigning new names only to those species which have at least been demonstrated with the electron microscope. In order to preserve continuity, however, it is suggested that at least for the time being all those species of insect viruses which have been previously recognized as such by Holmes in *Bergey's Manual of Determinative Bacteriology* be accepted and retained even though some of them may not as yet have been seen with the electron microscope or demonstrated by a determination of their physical properties.

Another discipline the writer would like to suggest concerns the description of the various species. In the sixth edition of *Bergey's Manual*, properties (*e.g.*, thermal inactivation) are listed for certain of the polyhedral viruses (genus *Borrelina*) which are based on studies made of polyhedral suspensions and not on the virus itself. Since significant amounts of virus are contained within the polyhedral body and protected by it, a determination of the thermal and other survival properties, for example, of such protected virus would not represent a true picture so far as the virus itself is concerned. It is therefore suggested that henceforth proper care be taken in citing the properties of the virus concerned to differentiate between free virus and virus incorporated within the polyhedral or other inclusion body. The same applies to those viruses of the genus *Bergoldia* which are surrounded by a proteinaceous envelope which undoubtedly exerts a protective influence.

Although aware of the faults inherent in using the hosts as criteria for differentiating species of viruses and in constructing keys to species, the writer recognizes the difficulty in using any other criterion, especially under the present circumstances, and for this reason has provisionally adopted the procedure used by Holmes (1948). For the sake of uniformity, in presenting the following classification we shall follow the generally accepted form used by *Bergey's Manual* wherever this form is applicable to our subject. In so doing, however, we are not acknowledging the necessity of including such matters as symptomatology in the descriptions; this information is being incorporated primarily for the convenience of the reader.

Order **VIRALES** Breed, Murray, and Hitchens
(J. Bact., **47**, 1944, 421)
Suborder **Zoophagineae** Holmes
(Bergey's Manual, 6th ed., 1948, 1225)

I. Viruses causing insect diseases characterized by the presence of polyhedral inclusions in the infected cells of the host. The inclusions (each of which contains numerous virus particles) originate in the nuclei of the host cells. The size and shape of the inclusions may vary within certain limits, but usually they are relatively uniform in these respects and in each case a more-or-less typical form ordinarily predominates.

Genus I. **Borrelina** Paillot

II. Viruses causing insect diseases characterized by the presence of refringent polymorphic inclusions of very irregular shape and size in the infected cells of the host. The inclusions originate in the cytoplasm of the host cell.

Genus II. **Paillotella** Steinhaus

III. Viruses causing insect diseases characterized by the presence, in large numbers, of very small but microscopically discernible granular inclusions in the infected cells of the host. The inclusions are particularly visible in the cytoplasm of the cells, but in some cases at least also occur in the nuclei. [In all instances so far known, the size of the inclusions is less than 1.0 micron in their longest diameter.] In all known cases, a single virus particle is located within each granule.

Genus III. **Bergoldia** Steinhaus

IV. Viruses causing insect diseases in which no visible pathological inclusion body of any kind is produced.

Genus IV. **Morator** Holmes

Genus I. **Borrelina** Paillot
(Compt. Rend. Acad. Sci., Paris, **182**, 1926, 182)

Viruses causing insect diseases characterized by the presence of polyhedral inclusions in the infected cells of the host. The inclusions (each of which contains numerous virus particles) originate in the nuclei of the host cells. The size and shape of the inclusions may vary within certain limits, but usually they are relatively uniform in these respects and in each case a more-or-less typical form ordinarily predominates.

The type species is *Borrelina bombycis* Paillot.

Key to the species of genus **Borrelina**[2]

I. Attacking the silkworm, *Bombyx mori* (Linn.)
1. *Borrelina bombycis* Paillot

II. Attacking the larva of the nun moth, *Lymantria monacha* Linn.
2. *Borrelina efficiens* Holmes

III. Attacking the larva of the gypsy moth, *Porthetria dispar* (Linn.)
3. *Borrelina reprimens* Holmes

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[2] Since this paper was written, Harriette B. Wasser and the writer have isolated the viruses causing polyhedrosis of the forest tent caterpillar, *Malacosoma disstria* Hbn., of the western tent caterpillar, *Malacosoma pluviale* (Dyar), and of the beet armyworm, *Laphygma exigua* (Hbn.). As determined from electron micrographs the size of the *M. disstria* polyhedron averages about 1.8 microns, while that of the virus particle is approximately 40 by 315 millimicrons. The polyhedron from *M. pluviale* averages about 1.6 microns in size, while the virus particle measures approximately 40 by 350 millimicrons. In the case of *L. exigua* the polyhedron has an approximate size range of 1.0 to 1.5 microns in diameter, and the virus particle is about 40 by 270 millimicrons in size. Recently, G. H. Bergold (Forest Insect Invest. Bi-monthly Prog. Rept., 1949, *5*, No. 3, p. 2) reported the isolation of a polyhedrosis virus from the spruce budworm, *Choristoneura fumiferana* (Clem.). The average dimensions of the virus particles are 28 millimicrons in width and 260 millimicrons in length.

IV. Attacking the western yellow-striped armyworm, *Prodenia praefica* Grote.
4. *Borrelina olethria* n.sp.
V. Attacking the alfalfa caterpillar, *Colias philodice eurytheme* Bdvl.
5. *Borrelina campeoles* n.sp.
VI. Attacking the California oakworm, *Phryganidia californica* Pack.
6. *Borrelina peremptor* n.sp.

Borrelina bombycis Paillot
(Compt. Rend. Acad. Sci., Paris, **182,** 1926, 182)

The following organisms and supposed organisms have been designated by their authors as the cause of silkworm jaundice: *Micrococcus bombycis* (Béchamp) Cohn, Beitr. z. Biol. d. Pflanzen, **1,** 1872, 165. *Microsporidium polyedricum* Bolle, Atti e Mem. dell'I. R. Soc. Agr. Gorizia, **33,** 1894, 193. *Micrococcus lardarius* Krassilstschik, Mém. Soc. Zool. de France, **9,** 1896, 513. *Chlamydozoon bombycis* von Prowazek, Arch. f. Protistenk., **10,** 1907, 363. *Crystalloplasma polyedricum* (Bolle) Prell, 1918, (see Verh. III Internat. Entomol.-Kongr., Zürich, **2,** 1926, 152.)

The Virus (Fig. 1): Rods, approximately 40 by 288 millimicrons, fairly uniform in size, occurring singly, in bundles of 2 and sometimes 4 units each. Comprise 3–5 per cent of polyhedron weight. Axial ratio 7.2; sedimentation constant s_{20} 1871; diffusion constant D_{20} 0.215 $\times 10^{-7}$; frictional ratio f/f_0 1.51. Particle weight (from $s_{20} - D_{20}$) 916 $\times 10^6$ (from length and diameter) 299 $\times 10^6$. Filterable through Berkefeld V and N, Chamberland L_1, L_2, L_3 filters. Chemical constituency: nucleoprotein (desoxyribonucleic acid type), relatively high phosphorus content. Inactivated (in polyhedral suspensions) by: boiling for 10 minutes, 60 C for 30 minutes, alcohol and ether, strong acids and strong alkalies, 1 per cent dodecyl sulfate, 36 per cent urea, 36 per cent guanidine, antiformin formalin. In polyhedral suspensions, resists action of weak acids and weak alkalies, drying, and is suspendible in water. (M.I.D. = 1.0×10^{-11} g/host animal.)

The Inclusion (Fig. 2): Polyhedron with 5 to 8 faces, usually 6 hexagonal rhombododecahedra; rather sharp, angular corners. Size ranges from 0.5 to 15 microns (average 3 to 5 microns) in diameter; usually fairly regular in size. Refractile crystallike, dense toward center, sometimes appears to have layers as though formed by accretion; may crack on pressure. Not optically active. Heavier than water. Stains rather poorly with ordinary aniline dyes; better when mordants are used. Has somewhat of a limiting "membrane." Soluble in weak alkalies (*e.g.*, 0.006M Na_2CO_3, 0.06 per cent KOH or NaOH; optimum solubility at pH

PLATE I

1. Electron micrographs of *Borrelina bombycis* Paillot, the cause of jaundice in the silkworm, *Bombyx mori* (Linn.). Top view shows individual virus particles, and bundles of two particles each; lower view (negative print) shows individual particles. The virus particles and bundles have been freed from the polyhedra by dissolution of the latter in dilute sodium carbonate. Approximate magnification, top view: 27,500 ×; lower view: 21,000 ×.
2. Polyhedra characteristic of silkworm jaundice, as seen with an ordinary light microscope. Magnification approximately 1,000 ×.
3. Electron micrograph of *Borrelina efficiens* Holmes, the cause of a polyhedrosis (*Wipfelkrankheit*) of the nun-moth caterpillar, *Lymantria monacha* (Linn.). Bundles predominate. Magnification approximately 16,800 ×.
4. Preparation showing the polyhedra characteristic of the polyhedrosis of the nun-moth caterpillar. Magnification approximately 460 ×.
5. Electron micrograph of *Borrelina reprimens* Holmes, the cause of a polyhedrosis ("wilt disease") of the gypsy-moth caterpillar, *Porthetria dispar* (Linn.). Magnification approximately 37,500 ×.
6. Polyhedra characteristic of the polyhedrosis of the gypsy-moth caterpillar. Magnification approximately 1,000 ×.

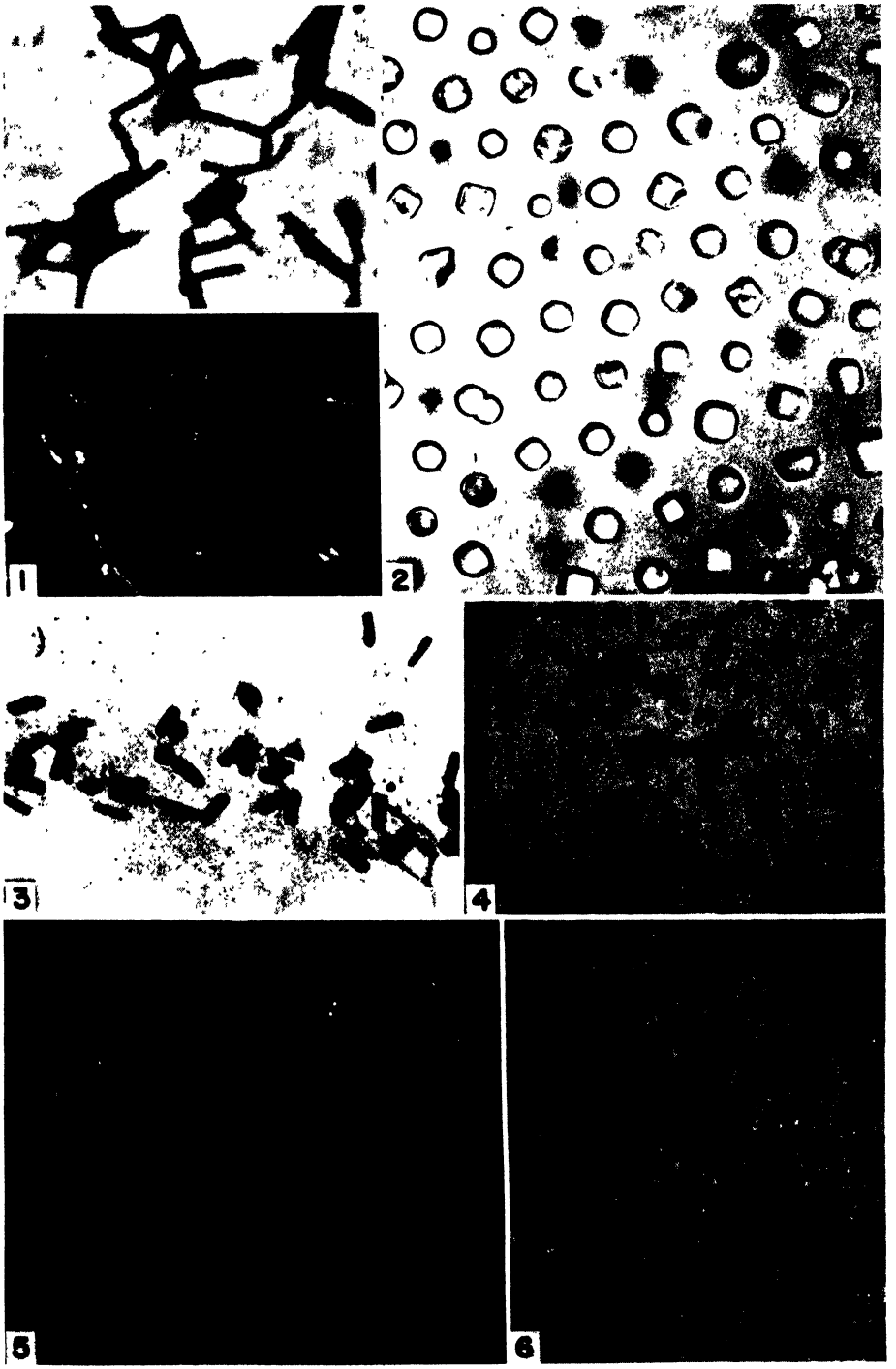

PLATE I

10.8 to 11.0) and in weak acids (optimum solubility at pH 1.0 to 0.5). Insoluble in hot or cold water, alcohol, chloroform, ether, or xylol. Chemical constituency: nucleoprotein. When dissolved in Na_2CO_3, the principal component has a molecular weight of about 378,000 and a diameter of about 10 millimicrons (sedimentation constant s_{20} 12.85); the molecular weight of the split components is about 60,500 (sedimentation constant s_{20} 3.16). Lower phosphorus content than virus. Contains no fat as indicated by its failure to stain with Sudan III and osmic acid. Amino acid content consists of following percentages; histidine 2.5, arginine 5.6, lysine 10.6, tyrosine 9.6, phenylalanine 6.7, tryptophan 3.3, cystine 0.52, methionine 3.3, and alanine 4.4. Small amounts of iron (0.5 per cent) are also detectable. The polyhedron protects virus activity from heat at 60 C for 30 minutes, sunlight irradiation 2 to 10 hours, putrefaction 22 months, 0 to 4 C in insect blood 1 year, storage in 1 per cent NaCl and glycerol in 1 per cent toluol, or in 1 per cent zepharol at 40 C for 22 months, drying in high vacuum (10^{-4} mm Hg) for 5 hours, drying in air at room temperature for months, and 15-minute treatments with the following: acetone, ether, 2.5 to 30 per cent formaldehyde, 5 per cent phenol, 5 per cent mercuric chloride, 70 per cent alcohol, and a 1:1 solution of 96 per cent alcohol and 1:1000 mercuric chloride. Virus activity completely inactivated when boiled in water for 10 minutes, or when treated for 15 minutes with trichloracetic acid.

Host: The silkworm, *Bombyx mori* (Linn.) (Lepidoptera, Bombycidae).

Disease Caused: Silkworm jaundice. (Synonyms: *grasserie, giallume, Gelbsucht.*) Symptoms: Infected larvae may exhibit loss of appetite and show general inactivity although frequently these symptoms are absent until late in the disease. The insect's body assumes a distended, swollen or fatlike appearance. Just before death the integument becomes opaque and assumes a shiny, yellow color. The larva is very flaccid because of the disintegration of internal tissues. The caterpillar is difficult to move without breaking the integument and liberating the liquefied contents. Incubation period 6 to 8 days. Polyhedra form in the nuclei of infected cells of hypodermis, fat body, tracheal matrix, and probably other tissues but not in the cells of the gut epithelium.

Pertinent Literature: Bergold, Biol. Zent., **63**, 1943, 1–55; Zeit. f. Naturforsch., **2b**, 1947, 122–143; Zeit. f. Naturforsch., **3b**, 1948, 25–26. Desnuelle, *et al.*, Ann. Inst. Pasteur, **69**, 1943, 75–86, 248–250; **71**, 1945, 264–272. Glaser and Chapman, Biol. Bull., **30**, 1916, 367–391. Glaser and Lacaillade, Am. J. Hyg., **20**, 1934, 454–464. Glaser and Stanley, J. Exp. Med., **77**, 1943, 451–466. Paillot, Traité des maladies du ver à soie, Doin, Paris, 1930, 137–184. Paillot and Gratia, Arch. f. Gesell. Virusforsch., **1**, 1939, 1920–1929. Steinhaus, Principles of Insect Pathology, McGraw-Hill, 1949, 425–449.

Borrelina efficiens[3] Holmes

(Bergey's Manual, 6th ed., 1948, 1226)

The following organisms and supposed organisms have at one time been designated by their authors as the cause of the polyhedrosis (*Wipfelkrankheit*) concerned: *Bacillus* "B" Hofmann, Die Schlaffsucht der Nonne, P. Weber, Frankfurt, 1891, 31 pp. *Bacterium monachae* v. Tubeuf, Forstl. Naturwiss. Z., **1**, 1892, 41. *Bacillus monachae* Eckstein, Z. Forst- u. Jagdwesen, **26**, 1894, 6. *Crystalloplasma monachae* Prell, Verh. III Internat. Entomol.-Kongr., Zürich, 1926, 152. By some authors: *Chlamydozoon prowazeki* Wolff, Mitt. Kais.-Wilh. Inst. Landw. Bromberg, **3**, 1910, 69 (named as causing polyhedrosis in larvae of *Bupalus piniarius* Linn.).

The Virus (Fig. 3): Rods, somewhat larger than *Borrelina bombycis* Paillot, occurring in bundles of 2 units each. Probably has the general physical and chemical properties similar to those of *Borrelina bombycis* Paillot.

The Inclusion (Fig. 4): Polyhedron more or less triangular to tetrahedral in shape. Size averages about 2.5 microns in diameter. Remaining characteristics similar to those described for polyhedra associated with *Borrelina bombycis* Paillot. The polyhedron protects

[3] As the result of an obvious typographical error, this specific epithet is spelled *efficiens* at the point where it is described. Elsewhere in the same publication it is spelled *efficiens*.

virus activity for 3 years when held in a dry state. Held moist, in glycerol, polyhedral suspensions retain virus activity for at least 5 days. Virus withstands putrefaction.

Host: Larva of the nun-moth, *Lymantria monacha* (Linn.) (Lepidoptera, Lymantriidae).

Disease Caused: Wipfelkrankheit (Synonyms: *Wipfelsucht*, nun-moth wilt, nun-moth polyhedrosis). Symptoms: Infected larvae show loss of appetite, become very flaccid, and if disturbed shortly before or after death the broken integument liberates the fluid, disintegrating internal tissues containing large numbers of polyhedra. Before dying the larvae tend to migrate to the tops of trees (*Wipfeln*). Incubation period from 13 to 15 days.

Pertinent Literature: Bergold, Biol. Zent., **63**, 1943, 1–55; Zeit. f. Naturforsch., **3b**, 1948, 25–26. Heidenreich, Arch. f. Gesell, Virusforsch., **1**, 1940, 582. Komárek and Breindel, Z. Angew, Entomol., **10**, 1924, 99–162. Wahl, Cent. Ges. Forstw., **35**, 1909, 164–172; 212–215; **36**, 1910, 377–397; **37**, 1911, 247–268; **38**, 1912, 355–378.

Borrelina reprimens Holmes

(Bergey's Manual, 6th ed., 1948, 1226)

At one time Glaser and Chapman (Science, **36**, 1912, 219) believed that the polyhedrosis of the gypsy moth was caused by a small gyrating micrococcus which they named *Gyrococcus flaccidifex*. A year later (J. Econ. Entomol., **6**, 1913, 479) they realized that this bacterium was only a secondary invader and that the true cause of the disease was a filterable virus.

The Virus (Fig. 5): Rods, approximately 41 by 360 millimicrons, occurring in bundles having an average size of 160 by 415 millimicrons. At certain points along each of the rod-shaped particles making up a bundle can sometimes be seen small nodes directly opposite each other when two or more particles are still hanging together. Sedimentation constant s_{20} 2500 to 4000 (depending on number of virus particles adhering together); diffusion constant D_{20} 0.175×10^{-7}; frictional ratio f/f_0 1.42; axial ratio 8.8. A particle weight of 1300×10^6 when calculated from the length and diameter of the virus particle as seen in electron micrographs. Filterable through Berkefeld N but apparently not through Chamberland F filters. Chemical constituency: nucleoprotein of the desoxyribonucleic acid type. (M.I.D. $= 1.0 \times 10^{-10}$ g/host animal.)

The Inclusion (Fig. 6): Polyhedron with 5 to 8 faces, crystalline in appearance, corners not regularly as sharp or as angular as in case of those associated with *Borrelina bombycis* Paillot. Average size 3.5 microns, although variations of from 0.5 to 15.0 microns have been reported. They are nucleoprotein in nature. The principal component has a sedimentation constant of s_{20} 12.57 and a molecular weight of 276,000. The split components have a sedimentation constant of 3.12 and a molecular weight of 47,250. In general properties, the polyhedra associated with *Borrelina reprimens* Holmes are very similar to those of the polyhedra associated with *Borrelina bombycis* Paillot (which see).

Host: Larva of the gypsy moth, *Porthetria dispar* (Linn.) (Lepidoptera, Lymantriidae).

Disease Caused: Polyhedrosis of gypsy-moth caterpillar. (Synonyms: wilt disease, gypsy-moth wilt.) Symptoms: Infected caterpillars lose their appetite, become sluggish in movement. Diseased insects usually seek an elevated place on their host plant. Just before death they become soft, the internal tissues disintegrate and liquefy, and upon rupturing the integument a brownish liquid is liberated.

Pertinent Literature: Bergold, Biol. Zent., **63**, 1943, 1–55; Zeit. f. Naturforsch., **2b**, 1947, 122–143; Zeit. f. Naturforsch., **3b**, 1948, 25–26. Chapman and Glaser, J. Econ. Entomol., **9**, 1916, 149–167. Glaser, J. Agr. Research, **4**, 1915, 101–128; Science, **48**, 1918, 301–302. Glaser and Chapman, J. Econ. Entomol., **6**, 1913, 479–488. Steinhaus, Principles of Insect Pathology, McGraw-Hill, 1949, 454–464.

Borrelina olethria n.sp.

(From Greek *olethrios*, destructive)

The Virus (Fig. 7): Rods, occurring in bundles of several members each. Approximate size 50 by 290 millimicrons.

The Inclusion (Fig. 8): Polyhedron, appears to have 4 or 5 sides when seen in outline. Size ranges from 2.0 to 5.0 microns with an average diameter of about 3.0 microns. General

properties probably similar to those described for the polyhedra associated with *Borrelina bombycis* Paillot.

Host: The western yellow-striped armyworm, *Prodenia praefica* Grote (Lepidoptera, Phalaenidae [Noctuidae]).

Disease Caused: Polyhedrosis. Symptoms: Infected larvae lose their appetites, become sluggish in movement. They may turn reddish brown before death, after which the body contents disintegrate into a dark watery mass within the integument.

Pertinent Literature: Blanchard and Conger, J. Econ. Entomol., **25**, 1932, 1059–1070. Steinhaus, Principles of Insect Pathology, McGraw-Hill, 1949, 467–468.

Borrelina campeoles n.sp.

(From Greek *kampe*, caterpillar + *olesai* to destroy)

The Virus (Fig. 9): Rods, approximately 40 by 300 millimicrons, occurring in bundles of several members each. Filterable through bacteriological filters of coarse to medium porosity. Most of the physical and chemical agents which destroy bacteria also destroy the virus. Incorporated within the polyhedron the virus is very resistant to drying and can retain its infectivity for at least a year.

The Inclusion (Fig. 10): Polyhedron, appears to have from 3 to 6 sides when seen in outline, varies considerably in shape. Corners are angular but somewhat rounded. Size varies from 1.0 to 3.0 microns in diameter, with an average width of about 1.5 microns. Exceptionally large polyhedra (4.0 to almost 5.0 microns) are seen occasionally. Most of the general properties of the polyhedra are essentially the same as those described under *Borrelina bombycis* Paillot.

Host: The alfalfa caterpillar, *Colias philodice eurytheme* Bdvl. (Lepidoptera, Pieridae).

Disease Caused: Polyhedrosis of the alfalfa caterpillar. (Synonyms: Wilt disease, wilt.) Symptoms: Infected larvae show loss of appetite, and decreased activity. The normally green color of the larva changes to a pale, yellowish, or grayish-green, sometimes giving the insect a mottled appearance. Body becomes very flaccid and somewhat darkened and usually dies about 7 days after infection. The internal tissues become liquefied and the caterpillar breaks down into a disintegrating mass. Pupa may also show symptoms of disease.

Pertinent Literature: Michelbacher and Smith, Hilgardia, **15**, 1943, 369–397. Steinhaus, J. Econ. Entomol., **41**, 1948, 859–865; Principles of Insect Pathology, McGraw-Hill, 1949, 477–484. Wildermuth, U. S. Dept. Agr., Circ. 133, 1911, 1–14; Bull. 124, 1914, 1–40.

PLATE II

7. Electron micrographs of *Borrelina olethria* n.sp., the cause of a polyhedrosis of the western yellow-striped armyworm, *Prodenia praefica* Grote. Top view shows virus bundles (dark bodies are salt crystals); lower view shows individual virus particles as seen in a gold-shadowed preparation. Magnification approximately 12,500 ×.
8. Polyhedra characteristic of the polyhedrosis of the western yellow-striped armyworm. Magnification approximately 1,000 ×.
9. Electron micrograph (gold-shadowed preparation) of *Borrelina campeoles* n.sp., the cause of a polyhedrosis of the alfalfa caterpillar, *Colias philodice eurytheme* Bdvl. Virus bundles as well as individual virus particles may be seen. Magnification approximately 12,500 ×.
10. Polyhedra characteristic of the polyhedrosis of the alfalfa caterpillar. Magnification approximately 1,000 ×.
11. Electron micrograph of *Borrelina peremptor* n.sp., the cause of a polyhedrosis of the California oakworm, *Phryganidia californica* Pack., showing individual virus particles. Magnification approximately 12,500 ×.
12. Polyhedra characteristic of the polyhedrosis of the California oakworm. Magnification approximately 1,000 ×.

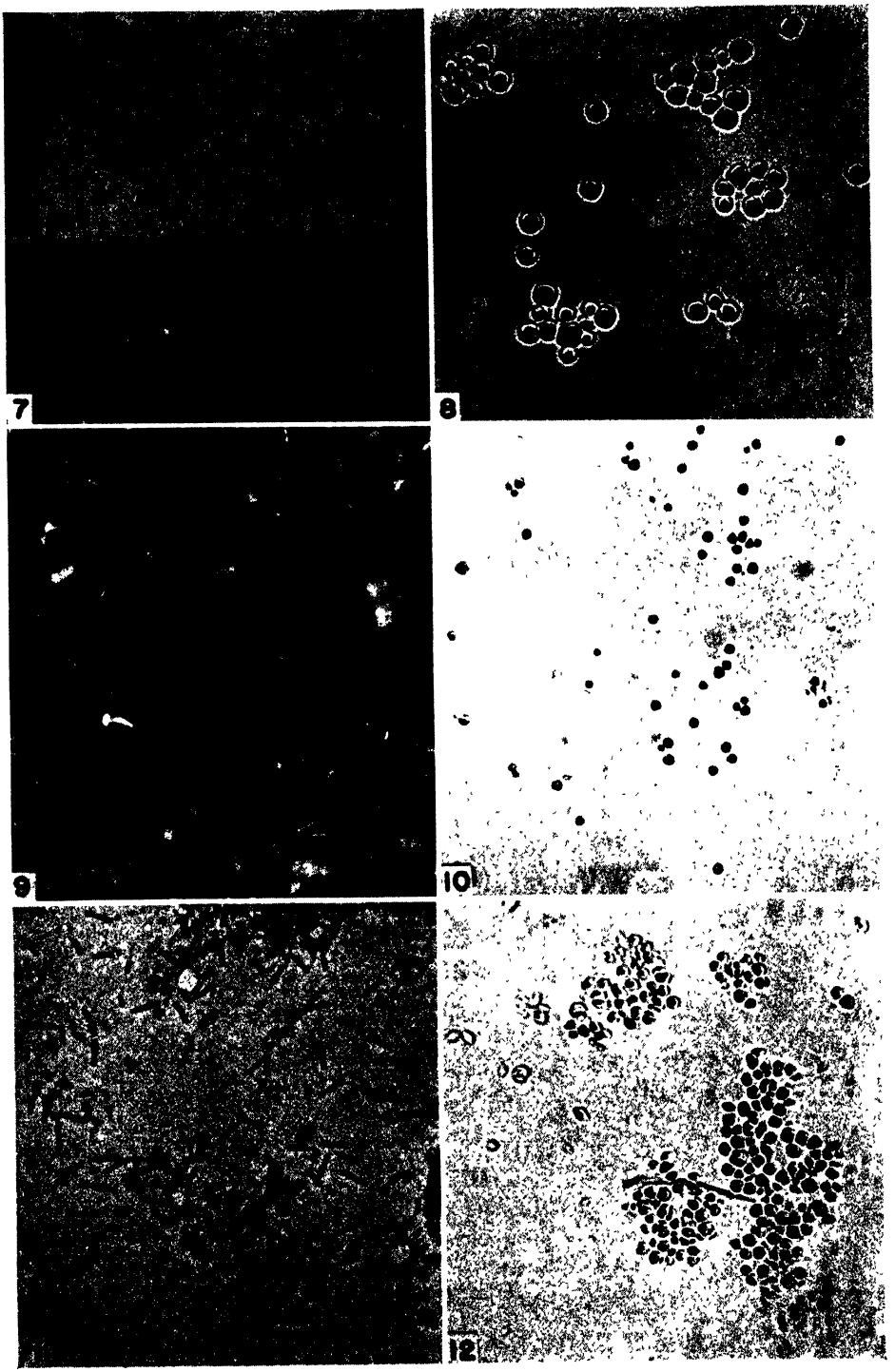

PLATE II

Borrelina peremptor n.sp.

(From Latin *peremptor*, a destroyer)

The Virus (Fig. 11): Rods, approximately 30 by 270 millimicrons, occurring in bundles of several members each (in electron microscope preparations so far examined, the bundle formation has been seen only rarely). Most physical and chemical agents which destroy bacteria also destroy the virus. Within the polyhedron the virus is protected from these agencies to a considerable degree.

The Inclusion (Fig. 12): Polyhedron, appears to have from 3 to 5 sides when seen in outline, varies considerably in shape and may be very irregular; corners rounded. Size ranges from 1.0 to 3.5 microns in diameter; averages about 2.0 microns.

Host: The California oakworm, *Phryganidia californica* Pack. (Lepidoptera, Dioptidae).

Disease Caused: Polyhedrosis of the California oakworm. Symptoms: Infected larvae become sluggish in movement and lose their appetites. Natural coloration becomes less intense; recently dead larvae may assume a pink or faint reddish color. Internal tissues liquefy; the darkened dead larvae lose their normal form and hang limp from host plant.

Pertinent Literature: Chapman and Glaser, J. Econ. Entomol., **8**, 1915, 140–150. Steinhaus, Principles of Insect Pathology, McGraw-Hill, 1949, 472.

Genus II. **Paillotella** Steinhaus

(Principles of Insect Pathology, McGraw-Hill, 1949, 422)

Viruses causing insect diseases characterized by the presence of refringent, polymorphic inclusions of very irregular shape and size in the infected cells of the host. The inclusions originate in the cytoplasm of the host cell.

The type, and only, species is *Paillotella pieris* (Paillot) Steinhaus.

Key to species of genus **Paillotella**

I. Attacking the larva of the cabbage butterfly of Europe, *Pieris brassicae* (Linn.)
1. *Paillotella pieris* (Paillot) Steinhaus

Paillotella pieris (Paillot) Steinhaus

(Compt. Rend. Acad. Sci., Paris, **182**, 1926, 182; Principles of Insect Pathology, 1949, 422 and 498.)

The Virus: Not yet demonstrated with certainty. Paillot has described certain small granules less than 0.1 micron in diameter which appear to be associated with infectiousness. Electron microscope observations not yet made. Granules retained by Chamberland filter of fine porosity and the infectivity of the blood is thus destroyed. Infectivity also destroyed by heating at 75 C for one-half hour.

The Inclusion (Fig. 22): Polymorphic. Refringent. Of irregular size and shape: annular, spherical, globoid, and elongated structures. Form in cytoplasm of infected cell. Believed to arise from the mitochondria of the cell. Occur in fat and blood cells.

Host: The larva of the cabbage butterfly of Europe, *Pieris brassicae* (Linn.) (Lepidoptera, Pieridae).

Disease Caused: Polymorphic-inclusion disease. Symptoms: Very few outward signs. The blood of diseased larvae is viscous and milky in appearance. Mortality varies, may be low to fairly high. Pupae also susceptible. Experimentally, virus difficult to establish in insects when given *per os*. Direct inoculation succeeds. Virus also transmitted from one generation to the next in association with the egg.

Pertinent Literature: Paillot, Compt. Rend. Acad. Sci., Paris, **182**, 1926, 180–182; Ann. Inst. Pasteur, **40**, 1926, 314–352. Steinhaus, Principles of Insect Pathology, McGraw-Hill, 1949, 497–500.

Genus III. **Bergoldia** Steinhaus

(Principles of Insect Pathology, McGraw-Hill, 1949, 422.)

Viruses causing insect diseases characterized by the presence, in large numbers, of very small but microscopically discernible granular inclusions in the infected cells of the host. The inclusions are particularly visible in the cytoplasm of the cells, but in some cases at least also occur in the nuclei. [In all instances so far known, the size of the inclusions is less

than 1.0 micron in their longest diameter.] In all known cases, a single virus particle is located within each granule.

The type species is *Bergoldia calypta* Steinhaus.

Key to the genus **Bergoldia**

I. Attacking the fir-shoot roller, *Cacoecia murinana* (Hbn.)
1. *Bergoldia calypta* Steinhaus

II. Attacking the variegated cutworm, *Peridroma margaritosa* (Haw.)
2. *Bergoldia daboia* n.sp.

III. Attacking the buckeye caterpillar, *Junonia coenia* Hbn.
3. *Bergoldia lathetica* n.sp.

IV. Attacking the salt-marsh caterpillar, *Estigmene acraea* (Drury)
4. *Bergoldia thompsonia* n.sp.

V. Attacking the larva of the cabbage butterfly of Europe, *Pieris brassicae* (Linn.).
5. *Bergoldia brassicae* (Paillot) Steinhaus

Bergoldia calypta Steinhaus

(Principles of Insect Pathology, McGraw-Hill, 1949, 422 and 512.)

The Virus (Fig. 13): Rods, 50 by 262 millimicrons, occurring singly and enclosed in a proteinaceous envelope or "capsule." Svedberg sedimentation constant s_{20} 1324; diffusion constant D_{20} 0.278 $\times$ 10^{-7}; a frictional ratio f/f_0 1.49; axial ratio 5.2. Particle weight 460 $\times$ 10^6 when calculated from sedimentation and diffusion constants, and 435 $\times$ 10^6 when calculated from the length and diameter of the particle as seen on electron micrographs.

The Inclusion (Fig. 14): Proteinaceous envelope or "capsule" which surrounds the virus particle. Approximate size: 0.23 by 0.36 micron. Dissolves in weak alkali giving a main component with a sedimentation constant of s_{20} 11.8, and a molecular weight of about 300,000. The split components have a sedimentation constant s_{20} 3.45, and a molecular weight of about 60,000.

Host: The fir-shoot roller, *Cacoecia murinana* (Hbn.) (Lepidoptera, Tortricidae).

Disease Caused: Granulosis. (Synonym: *Kapselvirus-Krankheit.*) Symptoms: Few external symptoms until shortly before death at which time the normal yellowish-green insects become thickly swollen and are colored a pale greenish hue. Upon puncturing the integument of a diseased larva, a milky-white fluid oozes out. This fluid contains the small granules which harbor the virus particles.

Pertinent Literature: Bergold, Zeit. f. Naturforsch., **3b,** 1948, 338–342. Steinhaus, Principles of Insect Pathology, McGraw-Hill, 1949, 510–514.

Bergoldia daboia n.sp.

(From Hindu *daboya*, that lies hidden)

The Virus: (Fig. 15): Rods, approximately 40 by 340 millimicrons, occurring singly and enclosed within ovoid envelope; occasionally somewhat curved. Sometimes several rods arrange themselves end to end in chainlike fashion.

The Inclusion (Figs. 16 and 21): Ovoid envelope covering virus particle, approximately 0.25 by 0.45 micron. Occasionally rather long coalesced forms seen. Soluble in low concentrations of Na_2CO_3.

Host: Variegated cutworm, *Peridroma margaritosa* (Haw.) (Lepidoptera, Noctuidae).

Disease Caused: Granulosis. Symptoms: Two or three days after infection the larvae begin to eat less food than normally; they remain slightly smaller in size than normally developing insects, have a somewhat languid appearance, and usually die before pupating. The larvae are flaccid but the body wall remains relatively firm. The fat tissue of the insect is especially affected. Histopathology of the disease under discussion is characterized by the accumulation of the granular inclusions in the diseased cell; they are particularly visible in the cytoplasm.

Pertinent Literature: Steinhaus, Science, **106,** 1947, 323–324; Principles of Insect Pathology, McGraw-Hill, 1949, 508–511. Steinhaus, Hughes, and Wasser, J. Bact., **57,** 1949, 219–224.

Bergoldia lathetica n.sp.

(From Greek, *lathētikos*, likely to escape notice.)

The Virus (Fig. 17): Rods, approximately 40 by 300 millimicrons, occurring singly and enclosed within an ovoid envelope.

The Inclusion (Fig. 18): Ovoid envelope, covering virus particle, approximately 0.30 by 0.45 micron. Soluble in low concentrations of Na_2CO_3.

Host: The larva of the buckeye, *Junonia coenia* Hübner (Lepidoptera, Nymphalidae).

Disease Caused: Granulosis. Symptoms: Similar to those caused by *Bergoldia daboia* in the variegated cutworm. The fat body of the buckeye is not as prominent as is that of the cutworm so that the white, opaque aspect of this structure is not as noticeable as in the latter insect. Buckeye caterpillars may be almost moribund without showing much external evidence of disease, except a general lack of activity and loss of appetite. Usually, however, the diseased larvae appear brownish in color and lose most of their blue metallic luster. Presence of the granules in the nuclei of the fat cells is more evident than in the cutworm. Granules may also be present in cytoplasm. The nuclei hypertrophy greatly before breaking down.

Pertinent Literature: Steinhaus, Principles of Insect Pathology, McGraw-Hill, 1949, 501. Steinhaus and Thompson, Science, 1949, **110**, 276–278.

Bergoldia thompsonia n.sp.

(Named for Clarence G. Thompson who first observed the infection in salt-marsh caterpillars collected in the field.)

The Virus (Fig. 19): Rods, approximately 40 by 270 millimicrons, occurring singly and enclosed within an ovoid envelope.

The Inclusion (Fig. 20): Ovoid envelope covering virus particle, approximately 0.25 by 0.40 micron. Soluble in low concentrations of Na_2CO_3.

Host: The salt-marsh caterpillar, *Estigmene acraea* (Drury) (Lepidoptera, Arctiidae).

Disease Caused: Granulosis. Symptoms: Loss of appetite and lessened general activity. The hairy covering of the larvae obscures any other external symptoms. Upon death the larvae are flaccid and soft.

Pertinent Literature: Steinhaus, Principles of Insect Pathology, McGraw-Hill, 1949, 501. Steinhaus and Thompson, Science, 1949, **110**, 276–278.

Bergoldia brassicae (Paillot) Steinhaus

(Paillot, Compt. Rend. Acad. Sci., Paris, **182**, 1926, 182. Steinhaus, Principles of Insect Pathology, McGraw-Hill, 1949, 502.)

The Virus: Not yet demonstrated with the electron microscope. Probably similar to the other viruses of this genus.

PLATE III

13. Electron micrographs (negative print) of *Bergoldia calypta* Steinhaus, the cause of a granulosis of the fir-shoot roller, *Cacoecia murinana* (Hbn.). Each of the virus particles has been freed from a granular inclusion body (as shown in fig. 14) by dissolution of the latter by dilute sodium carbonate. Magnification approximately 27,000 ×.
14. Electron micrograph (negative print) of the granular inclusions characteristic of the granulosis of the fir-shoot roller. The large form probably represents a combined grouping of granules. Magnification approximately 27,000 ×.
15. Electron micrograph of *Bergoldia daboia* n.sp., the cause of a granulosis of the variegated cutworm, *Peridroma margaritosa* (Haw.). Magnification approximately 12,500 ×.
16. Electron micrograph of the granular inclusions characteristic of the granulosis of the variegated cutworm. Magnification approximately 12,500 ×.
17. Electron micrograph of *Bergoldia lathetica* n.sp., the cause of a granulosis of the buckeye caterpillar, *Junonia coenia* Hbn. Magnification approximately 12,500 ×.
18. Electron micrograph (gold-shadowed preparation) of the granular inclusions characteristic of the granulosis of the buckeye caterpillar. Magnification approximately 12,500 ×.

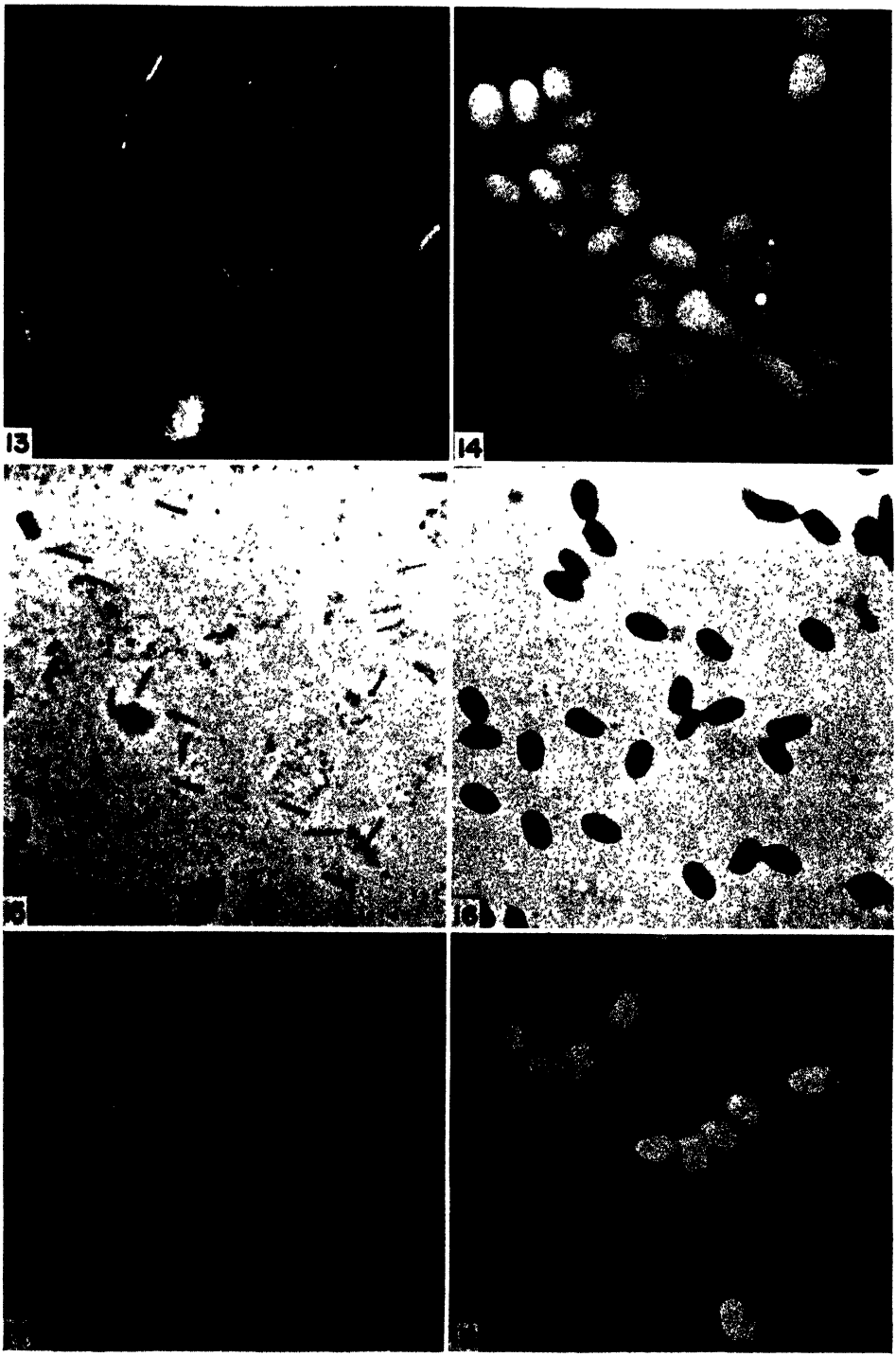

PLATE III

The Inclusion: Similar to that of other granuloses. Size averages about 0.2 to 0.3 micron "in diameter" (Paillot) although they are probably ovoid in shape as are the inclusions of the other granuloses.

Host: Larva of the cabbage butterfly of Europe, *Pieris brassicae* (Linn.) (Lepidoptera, Pieridae).

Disease Caused: Granulosis. Symptoms: Infected insects exhibit a whitish-yellow color on their ventral surface, lose their appetite and are less active. Upon death the body is soft and flaccid.

Pertinent Literature: Paillot, Compt. Rend. Acad. Sci., Paris, **182,** 1926, 180-182; *ibid.*, **198,** 1934, 204-205. Steinhaus, Principles of Insect Pathology, McGraw-Hill, 1949, 502.

Genus IV. **Morator** Holmes

(Bergey's Manual, 6th ed., 1948, 1227.)

Viruses causing insect diseases in which no visible pathological inclusion body of any kind is produced.

The type, and only reported species is *Morator aetatulae* Holmes.

Key to species of genus **Morator**

I. Attacking the honeybee, *Apis mellifera* Linn.
1. *Morator aetatulae* Holmes

Morator aetatulae Holmes

(Bergey's Manual, 6th ed., 1948, 1227.)

The Virus: Not yet demonstrated with the electron microscope.[4] Passes Pasteur-Chamberland and Berkefeld filters. Virus is rendered inactive when heated in water at 59 C for 10 minutes, and in honey at approximately 70 C for 10 minutes. It withstands drying at room temperature for approximately three weeks. Dried virus exposed to the direct rays of the sun is destroyed in from 4 to 6 hours; when it is suspended in honey it is destroyed in from 5 to 6 hours. When suspended in honey and shielded from the direct sunlight the virus remains virulent for slightly less than one month at room temperature during the summer. In the presence of fermentative processes taking place in a 10 per cent cane sugar solution at room temperature, the virus is destroyed in about 5 days—the same period of survival as in a 20 per cent honey solution at outdoor temperatures. In the presence of putrefactive processes the virus remains virulent for approximately 10 days. The virus will resist 0.5, 1.0, and 2.0 per cent phenol for more than 3 weeks. Dead larvae which have remained in the brood comb more than one month are usually noninfectious. A single, freshly dead larva, contains enough infectious material to kill at least 3,000 healthy larvae in one week. Trans-

[4] Working with certain purified materials from honeybee larvae dying of sacbrood, Harriette B. Wasser and the writer have obtained electron micrographs of small particulate bodies which may possibly represent the virus of sacbrood although their identity has by no means been proved. Similar bodies have not as yet been found in material from healthy larvae. On the other hand, the infectivity of the minute particles remains to be demonstrated. The particles are spherical to slightly oval in shape and are approximately 60 millimicrons in diameter.

PLATE IV

19. Electron micrograph of *Bergoldia thompsonia* n.sp., the cause of a granulosis of the saltmarsh caterpillar, *Estigmene acraea* (Drury). Two "granules" may also be seen. Magnification approximately 12,500 ×.
20. Electron micrograph (gold-shadowed preparation) of the granular inclusions characteristic of the granulosis of the saltmarsh caterpillar. Magnification approximately 12,500 ×.
21. Wet-mount preparation (as seen with an ordinary light microscope) of the granular

(Continued on opposite page)

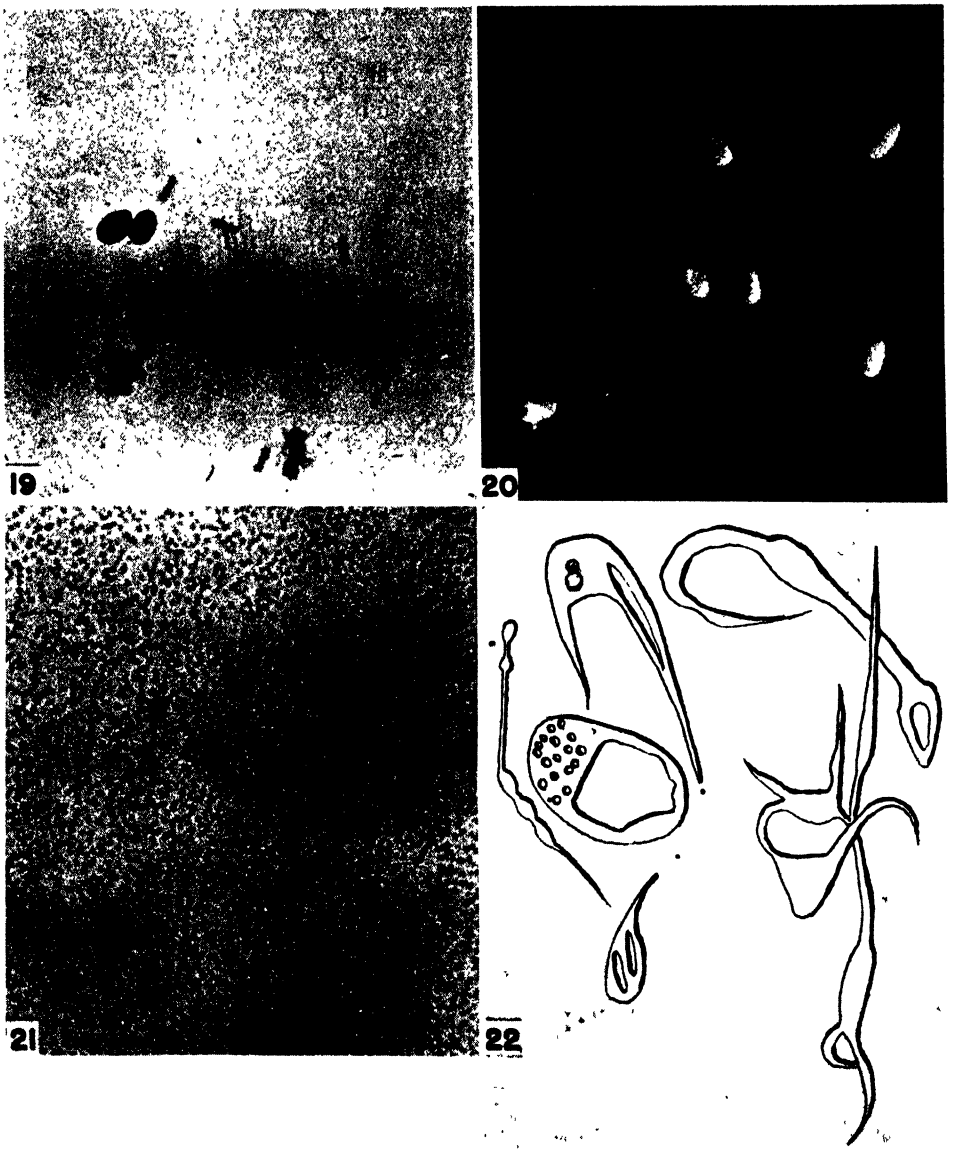

PLATE IV

inclusions from the fat tissue of a variegated cutworm, *Peridroma margaritosa* (Haw.), infected with *Bergoldia daboia* n.sp. Magnification approximately 650 ×.

22. Drawing of the refringent polymorphic inclusion bodies which characterize the disease of the larva of the cabbage butterfly of Europe, *Pieris brassicae* (Linn.), caused by *Paillotella pieris* (Paillot). (Redrawn from Paillot, 1926.)

(Photographs 1, 3, 5, 13, and 14, are from publications by Dr. G. H. Bergold and were obtained through the courtesy of Dr. Bergold. Photographs 2, 4, and 6 were obtained through the courtesy of the late Dr. R. W. Glaser. Photographs 7, 8, 9, 10, 12, 15, 16, 18, 20, and 21 were made for the author by Mr. K. M. Hughes; and photographs 11, 17, and 19 were prepared by Mrs. H. B. Wasser.)

mission occurs through the agency of contaminated food and probably water supply of insects.

Host: The larva of the honeybee, *Apis mellifera* Linn. (Hymenoptera, Apidae).

Disease Caused: Sacbrood, Symptoms: In his taxonomic account, Holmes (1948) described the disease as follows: "In the honey bee, immature stages only are susceptible; infected larvae die, usually after capping, some of the dead brood being uncapped by the bees. Occasionally caps are punctured. Affected areas of comb are usually small and scattered. Each larva is extended along its cell, head turned upward toward the roof. A larva recently dead appears light yellow, light gray, or light brown, soon darkening to brown or almost black. Cuticle of dead larva tough, permitting extraction of the saclike mass without rupture; contents watery with many suspended, fine brown particles. There are no characteristic intracellular bodies in affected tissues. Dead larvae eventually dry down to form scales that are black and roughened, that separate readily from the cell wall, and that may be lifted out intact."

Pertinent Literature: Bergey's Manual, 6th ed., 1948, 1227–1228. White, U. S. Dept. Agr., Bur. Entomol., Circ. 169, 1913, 1–5; U. S. Dept. Agr., Bull. 431, 1917, 1–54. Steinhaus, Insect Microbiology, Comstock Publ. Co., 1946, 426–430; Principles of Insect Pathology, McGraw-Hill, 1949, 514–523.

RECAPITULATION

Recent attempts to devise a satisfactory system of classifying plant and animal viruses have made it necessary to give further consideration to the systematic aspects of insect viruses if they are not to be left behind in the development of virus taxonomy. To assist in bringing this about, the writer has followed his previous proposal that the known insect viruses be separated provisionally into at least four generic groups (*Borrelina*, *Paillotella*, *Bergoldia*, and *Morator*) according to the type of inclusion body formed in the tissues of the diseased host, and the type of disease produced. In the present paper these groups have been further delineated, descriptions of species have been revised, and the following new species named: *Borrelina olethria*, *Borrelina campeoles*, *Borrelina peremptor*, *Bergoldia daboia*, *Bergoldia lathetica*, and *Bergoldia thompsonia*.

Although more than a hundred different insect hosts are known to be susceptible to probably as many different viruses, it is suggested that in the future no name be given to a virus until the agent has been demonstrated by physical or visual means in such a manner that at least its approximate size and shape are known. For instance, all new species described and named in the present paper have been demonstrated at least with the electron microscope. It is further recommended that the properties and characteristics of those insect viruses contained within inclusion bodies be determined on the basis of tests on the free virus rather than, as in the past, on the virus protected by the inclusion.

As the techniques of virus research are refined the characteristics and properties of the insect viruses will undoubtedly become more accurately known. The keys, descriptions, and groupings proposed in this paper are advanced as suggestions that may assist future virus systematists in better appraising the several kinds of viruses which cause infections in insects.

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THE GENUS PEDIOCOCCUS[1]

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A lactic acid producing sarcinalike organism has been associated with a type of spoilage in beer since the condition was first described by Pasteur (1876). He found that these organisms, which occur in pairs and fours and were sometimes associated with long rod bacteria, are the cause of the socalled "beer sickness." Their relationship to the true lactic acid bacteria of the genera *Lactobacillus* and *Streptococcus* was not realized until years later. The organism in spoiled beer was first referred to as a sarcina by Hansen (1879). The spoilage was studied by Balcke (1884*a*); and in Balcke's (1884*b*) second paper he recognized the assistance of Dr. Kurth in determining that the organism was not a true sarcina. Balcke applied the binomial *Pediococcus cerevisiae* to this species. In a series of papers, Lindner (1887, 1888, 1889) described the organisms involved in "beer sickness" more completely and proposed new specific names for types producing somewhat different reactions from that of the strain which he believed was *Pediococcus cerevisiae*.

The pediococci were not associated with other fermentations until years afterward, and it is doubtful whether anyone except Henneberg (1926) associated them with desirable fermentations such as those brought about by some species in the closely related genera *Streptococcus* and *Lactobacillus*. In this respect they might be compared with the genus *Leuconostoc*. The ability of species of *Leuconostoc* to bring about desirable changes was not recognized until Hammer (1923), Orla-Jensen (1919), and Pederson (1929, 1930) found that they were important in certain fermentations. The pediococci may be found to be very important in some vegetative fermentations when more is learned about their true role in nature.

The name "*Sarcina*" may continue to be attached to these organisms by brewing technologists but they should be classified properly by bacteriologists in relation to other organisms.

Since the name *Pediococcus* was first used by Balcke, this has been accepted and used as a generic name in connection with other specific names by Lindner (1887, 1927, 1928), De Toni and Trevisan (1889), Reichard (1894), Fischer (1897), Claussen (1903), Sollied (1903), Henneberg (1926), Pribram (1933), Mees (1934), and others. Buchanan (1925) states: "If the cocci which are arranged in tetrads are to receive generic recognition, the name *Pediococcus* would appear to be valid. The type species is *Pediococcus cerevisiae* Balcke."

In the 6th edition of Bergey's Manual (Breed *et al.*, 1948) the genus is included in an appendix to the family *Micrococcaceae*, with the type species *Pediococcus*

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cerevisiae Balcke. Twelve other species are listed as having been described. In regard to the genus, it is stated: "The following genus is recognized by workers in the brewing industry. It includes species that present characters intermediate between *Micrococcus*, *Sarcina*, and *Streptococcus*. Many students prefer to regard these as species of *Sarcina* (Macé, 1901)."

Still others (Lehmann and Neumann, 1896, Migula, 1900, Hucker, 1924) have felt that *Pediococcus* may be regarded as a synonym of *Micrococcus*. Buchanan (1925) stated that if *Pediococcus* does not receive generic recognition it perhaps may become a synonym of *Micrococcus*.

Beijerinck (1908) applied the name *Lactosarcina* to this genus in line with his names *Lactococcus* and *Lactobacillus*. Shimwell (1947) considers that they should be included among the plant types of the genus *Streptococcus*. Mees (1934) would include in the genus two strains described as *Tetracoccus* by Orla-Jensen (1919). Macé (1901) included these under *Sarcina*. Pribram (1933) placed the genus in a new family, *Pediococcaceae* and attributed the generic name to Balcke, but gave as the type *Pediococcus tetragenus* Koch and Gaffky. This as well as the other species named, *P. tetrus*, *P. flavus* and *P. gadidarum*, apparently do not belong in the genus.

Lindner credited Hayduck (1885) with a study of two sarcinalike organisms. Lindner studied two types of organisms, the first a low acid producer which he considered to be a strain of *Pediococcus cerevisiae*. The second produced more acid and was thus given the name *Pediococcus acidi lactici*. The acid was identified as lactic acid. The optimum temperature of growth was found to be 41 C. In a second paper Lindner (1888) described another species, *Pediococcus albus*. In this second study, reference is made to the fact that he studied the strain of *Pediococcus cerevisiae* that von Huth had studied. However, according to Mees (1934) von Huth studied an organism from horse urine which was believed to be the source of the organism in beer. There seems to have been no definite study of the original culture of Balcke and thus Lindner was possibly wrong in calling von Huth's culture *Pediococcus cerevisiae*. Lindner (1889) applied a third name *Pediococcus viscosus* to a slimy variety.

De Toni and Trevisan (1889) accepted the generic name and listed the first three specific names mentioned above and three others, *Pediococcus kochii*, *Pediococcus maggiorae*, and *Pediococcus aurantiacus*. However, they credited the name to Lindner and list *Pediococcus acidi-lactici* first. These three other species are different and should not be included in the genus. A number of other names have been applied to sarcina like organisms in beer, including *Pediococcus sarcinaeformis* by Reichard (1894), *Pediococcus damnosus* and *Pediococcus perniciosus* by Claussen (1903), *Pediococcus kiliensis* by Bettges (1906), *Pediococcus odoris mellisimalis* and *Pediococcus acidulefaciens* by Schönfeld (1904), *Pediococcus lindneri* by Henneberg (1926), and *Pediococcus halophilus*, *Pediococcus pentosaceus*, and *Pediococcus urinae equi* by Mees (1934). Many of these early studies centered around the technique of isolating the organisms in pure culture and of course also involved the particular type of beer. Sollied (1903) isolated a strain from a potato mash and applied the name *Pediococcus hennebergi*. This

apparently was the first type isolated from a product other than beer or beer mash and it differed from all of the others except possibly *Pediococcus acidi-lactici* in that it had a higher optimum temperature for growth, 40 C, and was an active arabinose fermenter. It produced inactive lactic acid. The pediococci have been studied by Hansen (1890), Brown and Morris (1895), and Reichard and Riehl (1895) some of whom have referred to them as sarcina.

The names *Pediococcus damnosus* and *Pediococcus perniciosus* were included in Lehmann and Neumann (1927), but no reference was made to the names proposed by Balcke or by Lindner.

Henneberg (1926) studied cultures isolated from grain mash and potato mash and observed them in sauerkraut, molasses, salt beans, and in pickle fermentations. In addition, he referred to the tetracocci of Orla-Jensen (1919). He included three species, *Pediococcus acidi-lactici* of Lindner, *Pediococcus hennebergi* of Sollied, the wild lactic acid bacteria of potato mash, and *Pediococcus lindneri*, a new type which he stated he originally confused with Lindner's *Pediococcus acidi-lactici*. He also discussed briefly a slime producing variety from kraut which produced gas and less acid than the other types and which he stated might be used as a starter for sauerkraut since it produced a good aroma. He noted that this type sometimes occurred in short chains. It seems obvious, however, that he had confused the pediococci with leuconostocs, a type whose importance in kraut fermentation he had not recognized. Henneberg's separation of species is based upon colony and morphological differences, utilization of different sugars, and the amounts of acid produced. He pointed out that they all produce inactive lactic acid with a trace of volatile acid.

Mees (1934) presented a historical review of the studies on the spoilage of beer by these organisms. Mees (1934) studied eight strains that he had isolated by various methods as well as strains of the genus *Tetracoccus* Orla-Jensen (1919), a culture he had received from van Niel, a culture from horse urine, and several others which eventually he decided were not of this genus. He noted that the cultures considered to be pediococci were all nonmotile, gram positive, nonspore-forming, catalase negative, spherical bacteria. On acid media a strong tendency toward tetrad formation was observed, but in neutral media they were mostly diplococci. Complex protein degradation products were required for growth. With the exception of one of the tetracoccus cultures, all cultures produced inactive lactic acid, grew best at 23 to 24 C, and readily fermented glucose, fructose, maltose, and galactose. Lactose, sucrose, xylose, and arabinose were utilized only to a limited degree, if at all. Mees accepted the generic name *Pediococcus*, but used *Pediococcus damnosus* Claussen as his type with *Pediococcus cerevisiae* Balcke as a questionable synonym. He characterized *Pediococcus perniciosus* Claussen as a variety of *P. damnosus* and named a new variety *P. salicinaceus*. Species names, *Pediococcus pentosaceus* and *Pediococcus halophilus*, were applied to organisms included by Orla Jensen (1919) in *Tetracoccus*. Still another name *Pediococcus urinae equi* was given for the type isolated from horse urine.

In a series of papers, Shimwell and Kirkpatrick (1939) and Shimwell (1940,

1947, 1948) have reviewed the subject of beer disease organisms and conclude that the beer sarcina or pediococci should be accepted in the plant division of the genus *Streptococcus* because they are not regarded as true sarcinae or as micrococci. Shimwell (1940) credits Mees with the first scientific study of the problem. Shimwell and Kirkpatrick pointed out that the production of lactic acid with a small amount of volatile acid and carbon dioxide, and the production under certain conditions of diacetyl (the aromatic substance of butter) are all characters of the streptococci. The fact that the organisms produce inactive lactic acid and occur in tetrad formation is not considered sufficient cause for a generic separation of these organisms from streptococci. Accordingly they consider the beer cocci to be intermediate, in the classification of Davis (1936), between the *dextro*-lactic acid producing *Streptococcus lactis* group and the heterofermentative *levo*-lactic acid, diacetyl producing vegetative type of the aroma group. Shimwell proposed a first species differentiation based on temperature optimum and a second one on sugar fermentation.

Recent observations. The pediococci were observed in this laboratory in microscopic preparations from fermenting sauerkraut a number of years ago. However, they apparently do not play an important part in a normal kraut fermentation and actually were not isolated until 1944 when five strains were obtained. Later, 49 cultures were isolated from fermenting beans in salt brine and 645 from fermenting salt stock cucumbers. They played an important part in the fermentation of the beans and in some of the series of cucumber fermentations, particularly in the 1946 series in those brines containing 2½ to 5 per cent of salt. All cultures were isolated from small, round or lens shaped colonies and grew well in a stab of glucose yeast extract tryptone agar. After preliminary studies, 121 representative cultures were selected for a more complete study. These were remarkably similar. They were gram positive, nonmotile, catalase negative coccus forms which tended to occur in packets of four cells. None of the cultures liquefied gelatin or produced indole. Using the same 0.5 per cent tryptone, 0.3 per cent yeast extract base with added buffer salts, no significant variation was noted in regard to the fermentation of sugars and the related carbon compounds.

The cultures varied somewhat in regard to the amount of acid produced in sugar media. All cultures produced from 0.5 to 0.9 per cent acid in glucose, fructose, mannose, galactose, and maltose with an approximate average or frequency distribution peak between 0.6 and 0.7 per cent. Final hydrogen ion concentration was high, pH 3.25 to 3.4 compared with 3.05 to 3.25 for cultures of homofermentative lactobacilli and 3.7 to 3.9 for high acid producing streptococci. The majority of the cultures fermented sucrose, lactose, raffinose, salicin, and amygdalin, the frequency distribution curve having a peak at 0.5 to 0.6 per cent. The majority of the cultures also fermented arabinose but the frequency peak was lower, 0.2 per cent, and the maximum acidity 0.6 per cent. Xylose in the same way supported a slight growth but only 20 per cent of the cultures produced more than 0.1 per cent acid, although 0.6 per cent was the maximum. Rhamnose was utilized to a slight degree by 35 per cent of the cultures, but the maximum acidity was 0.3 per cent. Nearly all cultures, 96 to 100 per cent failed to utilize mannitol,

alpha-methylglucoside, inulin, dextrin, or starch. One culture produced 0.54 per cent acid in inulin. In all other cases, 0.2 per cent acid was the maximum produced.

All cultures selected for the study produced inactive lactic acid with a trace of volatile acid and a small amount of carbon dioxide, approximately 2 per cent of the sugar utilized. All cultures produced their maximum acidity between 22 and 32 C, but they also produced acid at 7 C and at 45 C. Some growth occurred in broth containing 10 per cent salt. However, the amount of acid produced increased as the salt concentration was decreased. In other words, salt does have some inhibitory action upon acid production at even 2 per cent concentration. The cultures varied in regard to growth in litmus milk. None grew rapidly; some curdled milk after a week at 32 C, but others failed to produce more than a slight acidity in two weeks.

On the basis of these results it would seem that there may be some differences between the various cultures which may be used in differentiating species. However, although one could select many strains which showed differences in regard to carbon compounds fermented, in no case could any significant trend be demonstrated. Therefore, one must conclude that all strains should be considered as belonging to one species.

In the isolation and study of these organisms from fermenting vegetables, 1438 strains of *Lactobacillus plantarum*, 282 strains of *Lactobacillus brevis*, 602 strains of *Leuconostoc mesenteroides*, and 783 strains of *Streptococcus faecalis* and closely related streptococci were also isolated and identified. Even though the pediococci are coccus forms, they were not confused morphologically or physiologically with the streptococci or leuconostocs at any time. The fact that the pediococci produced approximately twice as much or more lactic acid than either of the other coccus types was sufficient in itself to distinguish between them. But, in addition, morphologically they are more rounded like the micrococci, and tetrad grouping was very common. With respect to the acidity produced in sugar media, they were more often confused with the gas forming lactobacilli and with the low acid producing strains of the non-gas forming lactobacilli. They could, however, be readily distinguished by microscopic examination. Thus one might say that in respect to acid production, they are intermediate between the homofermentative lactobacilli and the streptococci. Little difference was found between the many isolates from widely differing sources such as fermenting beans which are fairly high in protein, fermenting sauerkraut, and cucumber pickles in 2½ to 5 per cent salt. It therefore seems very doubtful that the different strains from beer vary sufficiently to warrant the recognition of a large number of species.

Concluding comments. Lindner, on the basis of a study of von Huth's culture from horse urine assumed that the organism studied by Kurth and named by Balcke was a low acid producing strain. There is no evidence that Lindner had Balcke's culture or one similar to it. The fact that it was assumed at that time that the pediococci may have been introduced from the stable is not sufficient reason to consider that the organism isolated from horse urine was the same as

that in the spoiled beer. Until such time that a sufficient difference can be noted between the various strains showing an optimum temperature range for growth between 20 and 30 C, and which include the strains described by Lindner, Claussen, Mees, and others, it seems most logical to consider that they are all similar to the original strain of *Pediococcus cerevisiae* Balcke or the slimy variety, *Pediococcus viscosus* Lindner.

Lindner's *Pediococcus acidi-lactici* and Sollied's *Pediococcus hennebergi* which apparently have an optimum temperature growth range at about 40 C and the two strains of tetracocci of Orla-Jensen may differ sufficiently to be considered as other species. Henneberg mentions pediococci of sauerkraut which he considered different from the other species.

The lactic acid producing cocci responsible for the socalled "sarcina sickness of beer" were given the name *Pediococcus cerevisiae* by Balcke (1884*b*). They are present in other fermenting materials in some of which they may have an important part in the fermentation. They are readily distinguished from the species of the genus *Streptococcus* by their tetrad grouping and their comparatively high acid production. These morphological and physiological characters and the fact that they produce inactive lactic acid seem sufficient to exclude them from the genera *Streptococcus*, *Micrococcus*, or *Sarcina*. The group should be considered as a separate genus, *Pediococcus* Balcke with the species *Pediococcus cerevisiae* Balcke as the type. There is no justification for the selection by Mees (1934) of *Pediococcus damnosus* Claussen, or by De Toni and Trevisan (1889) of *Pediococcus acidi lactici* Lindner as the type. Although it is granted that Balcke did not give a complete description of either his genus or species, they were well enough described so that other workers have recognized the organisms. The studies of Lindner (1887, 1888, 1889) further identified the organisms, and they were well recognized long before the work of Claussen (1903). The genus should be included in the tribe *Streptococceae* of the family *Lactobacteriaceae* with the genera *Diplococcus*, *Streptococcus*, and *Leuconostoc*, rather than in the family *Micrococcaceae* as in Bergey's Manual (1948).

The genus should include those gram-positive nonmotile, nonsporeforming cocci that occur in tetrads and sometimes singly or in pairs, show poor surface growth because they are microaerophilic, are high acid homofermentative lactic acid producers and do not reduce nitrates, liquefy gelatin or produce catalase.

The type species is *Pediococcus cerevisiae* Balcke. The species produces inactive lactic acid from sugars, always fermenting glucose, fructose, mannose, galactose, maltose and usually arabinose, sucrose, lactose, raffinose, salicin and amygdalin and, sometimes to a lesser degree, xylose and rhamnose. Nearly all strains fail to ferment mannitol, *alpha*-methylglucoside, inulin, dextrin, and starch. Milk may be fermented slowly by some strains. The optimum temperature range for fermentations is between 25 and 32 C. The organism was first observed in "sarcina-sick" beer but may occur in the fermenting materials such as sauerkraut, or pickles.

If a slimy variety is definitely shown in the future to be distinct enough to be given species or varietal recognition, the name proposed by Lindner, *Pediococcus*

viscosus has priority over other names. Similarly if a type is shown in future studies to have the higher optimum temperature for growth and warrants species or varietal recognition *Pediococcus acidi-lactici* Lindner or *Pediococcus hennebergi* Sollied may be recognized.

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CLOSTRIDIA IN GAS GANGRENE

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The rapidly progressive infections of muscle brought about by some members of the genus *Clostridium* have been investigated by a number of workers during the past ten years. In general, such investigations fell into three groups: investigations of the clostridia found in war wounds and the factors that affect their growth; investigations of the exocellular toxins produced by the pathogenic clostridia and the relation of these toxins to virulence; and investigations of the effect of chemotherapeutic agents on clostridia.

Local defense mechanisms of the host appear to play no part in gas gangrene—these infections seem to be primarily the result of an actively growing organism invading a relatively passive host. Much interest has been shown in the toxic exocellular products of the pathogenic clostridia, for the activity of the organisms is largely a reflection of the exocellular toxins produced by them. With regard to the role that such products play in these infections, MacFarlane and Knight (48) have pointed out that in searching for biochemical mechanisms by which bacterial toxins affect the cells of the host, two main hypotheses should be considered: first, the toxin may be a substance which blocks a metabolic reaction of the host by interfering with an essential enzyme system; and second, the toxin may be an enzyme itself, exerting its toxic action by attacking one or more specific substrates which are normal constituents of the cells of the host and which are accessible to the enzyme. Gale (30) has presented an excellent discussion of *C. perfringens* infections from this point of view.

Since the progress of these clostridial infections seems to be governed more by the properties of the invading organisms than by the resistance of the host, it was thought that it would be interesting and perhaps informative to consider gas gangrene primarily with regard to the bacteria involved, and the factors which affect their growth in the animal body. The surgical literature on gas gangrene has been reviewed by Altemeier and Furste (5).

This review is restricted, largely, to investigations carried out since the beginning of the second World War, a period when the study of clostridial infections was given impetus by the large number of cases developing in battle casualties. It was deemed desirable to limit consideration of human cases to those arising from wounds of warfare, for it was only in active theaters of war that gas gangrene occurred with sufficient frequency to make valid comparisons possible.

CLOSTRIDIA FOUND IN GAS GANGRENE AND THEIR ORIGIN

Several investigators have attempted to isolate and identify the sporeforming anaerobic bacteria found in gas gangrene. A summary of their results is given in table 1, which, however, does not include strains designated by the authors

as "unidentified". It is apparent that many species other than those deemed pathogenic can grow in tissue which has been invaded by members of the pathogenic species, or which has been rendered anoxic by impaired blood supply. Although there was considerable variation so far as individual species were concerned, the over-all isolation rate of the various workers was surprisingly uniform: MacLennan (51) found an average of 2.62 species of clostridia per case, Stock (95) an average of 2.56, and Smith and George (91) an average of 2.84. The incidence of *Clostridium novyi*[1] found by all of these workers was higher than was expected on the basis of the results of investigations made during the first World

TABLE 1

Clostridial Flora of Gas Gangrene

ORGANISM	PER CENT OF CASES			
	MacLennan (51) (146 cases)	MacLennan (52) (17 cases)	Stock (95) (25 cases)	Smith and George (91) (110 cases)
C. perfringens	56	83	80	39
C. novyi	37	47	48	32
C. septicum	19	24	4	
C. histolyticum	6	6		
C. tetani	13		8	4
C. bifermentans	4	35	20	54
C. sporogenes	37	50	72	54
C. tertium	30	59	8	3
C. multifermentans				5
C. butyricum	13		4	3
C. capitovale	5			3
C. fallax	1		4	3
C. cochlearium	9		4	2
C. putrificum	19			2
C. regulare				2
C. sphenoides	3			2
C. paraputrificum				1
C. hastiforme	3			
C. tetanomorphum	2			

War. On the other hand, the incidence of *Clostridium septicum* reported by Stock and by Smith and George was strikingly low. These workers considered the methods they used to be adequate for the isolation of this organism. *Clostridium histolyticum* was also much less common than was expected. *Clostridium feseri* was not isolated by any investigator. Although this species is pathogenic for domestic and laboratory animals, it appears that it is not pathogenic for man. The high incidence of *Clostridium sporogenes* and *Clostridium bifermentans* in cases of gas gangrene encountered in Sicily and Italy leads one to expect that this might be associated with the occurrence of the organisms in the soil.

[1] For the sake of uniformity, the nomenclature used throughout this review is that of the sixth edition of Bergey's Manual of Determinative Bacteriology.

Indeed, it appears likely that many of the clostridia found in wounds of warfare have their origin in the soil. The remainder probably originate from fecal contamination of the wounds. While it is impossible in any given case to ascertain the source of the organisms, the relation between the incidence of clostridial infections and the terrain over which the fighting took place indicates that soil is involved. MacLennan (51, 52) in an excellent series of studies on clostridial infections emphasized this relation with regard to the incidence of gas gangrene in the British Eighth Army. For a considerable time the fighting took place on the almost sterile sand of the Sahara desert; during this period the incidence of gas gangrene was 3.4 per 1000 wounded. When the fighting moved to the more

TABLE 2

Clostridia Isolated from Sand or Sandy Soil in North Africa (From MacLennan (51))

ORGANISM	NUMBER OF TIMES ISOLATED		
	Desert (41 samples)	Soiled desert (24 samples)	Cultivated areas (26 samples)
C. perfringens	1	6	22
C. novyi	1	0	8
C. septicum	0	0	4
C. tetani	1	1	6
C. histolyticum	0	1	0
C. sporogenes	2	8	16
C. putrificum	1	0	3
C. capitovale	1	0	0
C. cochlearium	0	1	5
C. tertium	0	3	9
C. butyricum	1	2	8
C. sphenoides	0	1	2
C. tetanomorphum	0	2	1
C. caloritolerans	0	1	0
Unidentified species	2	4	7
Average number per sample	0.27	1.2	3.5

fertile soil of Tripolitania and Tunisia, the incidence of gas gangrene increased to approximately 6 to 7 per 1000. The clostridia isolated from soil of these areas by MacLennan are listed in table 2. The column headed "soiled desert" refers to specimens obtained from desert roads and tracks and from the site of encampments still or recently occupied.

MacLennan believed that the organisms responsible for the infection of most of the wounds sustained in the desert fighting had their origin by direct or indirect fecal contamination of the wounds. That men fighting in the desert carried a considerable population of clostridia with them is indicated by MacLennan's investigation of clothing. The pieces of cloth investigated were cut from the clothing of wounded men, incubated for 48 hours in tubes containing cooked meat medium and then examined for the presence of clostridia. The results, given in table 3, indicate not only that clostridia can be easily isolated from such a

source, but also that the woolen battledress did not harbor significantly more clostridia than did the cotton khaki drill. This point is emphasized because of the opinion held by some surgeons that woolen clothing selectively harbors anaerobes (5, 54, 77). One observer (29) considered that the contamination of the woolen cloth did not come from contamination of the cloth as such, but occurred while the wool was on the sheep.

Miles, *et al.* (64), in an investigation of the bacterial flora of wounds of combat and those incurred in air raids, pointed out that the incidence of *C. perfringens* in wounds suffered by men in combat was about five times as high as the incidence in wounds of civilian air raid casualties (22.8% compared to 4.1%). *C. sporogenes* was found in 10.5% of military wounds and 4.1% of air raid wounds. The higher incidence of clostridia in wounds of combat troops may well be a

TABLE 3

Clostridia Isolated from Clothing

(From MacLennan (51))

ORGANISM	NUMBER OF TIMES ISOLATED*			
	Battledress		Khaki drill	
	Tunics	Trousers	Shirts	Shorts
C. perfringens	6	11	10	29
C. novyi	0	1	0	0
C. septicum	0	0	0	1
C. histolyticum	0	0	0	0
C. bifermentans	0	0	1	0
C. fallax	0	0	0	0
C. sporogenes	4	7	5	9
Other clostridia	11	12	8	6
Totals	21	31	24	45

* From 25 samples of each class of garment.

reflection of soil contamination, for the intimate contact of men in the infantry with dust in the dry season and mud in the wet season can hardly be overemphasized.

The report of Ferguson (26) on casualties sustained in amphibious operations in island warfare in the central Pacific also indicated a close association between soil contamination and the incidence of gas gangrene. The patients he was concerned with included 2664 wounded men; 1229 Navy, 1598 Marine, and 506 Army casualties. These casualties did not include burns. Almost all of the Army and Marine casualties were from land operations, those from the Navy from naval operations. Of 31 possible cases of gas gangrene, all save one were from land casualties.

On the continent of Europe, the incidence of gas gangrene was as high as it was during the first World War. During 1917–1918, the incidence was 3.6 per 1000 (51), while for World War II Langley and Winkelstein (43) reported 9.5

per 1000 hospital admissions; Odom (72) reported 445 cases in 92,030 casualties in the U. S. Third Army, an incidence of 4.8 per 1000; and the Dieppe raid yielded an incidence of 30 per 1000 (68). In the island warfare of the central Pacific, the incidence was also high, although somewhat more variable. Ross and Ryan (86) reported an incidence of 45 per 1000 in the Owen-Stanley and Buna-Gona campaigns, and Neel and Cole (68) reported an incidence in amphibious warfare of 7 per 1000. In 160 cases with severe wounds, the latter workers (69) found 27 cases of gas gangrene, an incidence of 169 per 1000 in these selected cases. That the incidence of gas gangrene was not uniform from one island to another is indicated by the report of Potts (76) who encountered no cases in one evacuation hospital.

The only report in which a high incidence of gas gangrene was noted in men who were not exposed to intimate contact with the soil is that of Cutler and Sandusky (20) who reported 18 cases per 1000 in wounds suffered in aerial combat.

The opinion is often expressed that the normal habitat of the pathogenic clostridia is the large intestine of man and animals and that these organisms exist in the soil only as spores. However, the author of this review has been unable to find, in the literature available to him, any evidence in support of this opinion. The results of the investigation of Smith and Gardner (92) indicate that *C. perfringens* does not always exist in the soil solely in the form of spores. These workers determined the numbers of heat susceptible (78 C for 20 minutes) and heat resistant forms of *C. perfringens* in soil which had not been subjected to recent fecal contamination and found that the majority of organisms were heat susceptible. They concluded that these probably were vegetative cells and that active growth of *C. perfringens* probably took place in the soil. The comparatively large numbers of organisms (from 100 to 50,000 per gram) found by them also would be difficult to explain on the basis of fecal contamination. Wan (100) reported that *C. perfringens* spores were found in numbers as high as 10,000 per gram of soil, but gave no information as to how his data were obtained.

The bacteriology of war wounds has been reviewed by Altemeier (2, 3). Two studies of the anaerobic bacterial flora of war wounds have been made recently by MacLennan (51) and Rustigian and Cipriani (87). The results of these authors are given in table 4. It should be noted that those of MacLennan were derived from a study of wounds sustained in the North African desert; consequently, the differences in the incidence of various species may be due, in some measure, to the material contaminating the wound originally. MacLennan particularly emphasized the relatively low incidence of proteolytic species in wounds incurred in the desert.

The bacteria, other than clostridia, found in gas gangrene do not differ appreciably from those found in ordinary contaminated wounds, except, perhaps, for lower incidence of some species. None of the workers in World War II reported the non-clostridial bacteria encountered in the cases of gas gangrene they investigated, but Smith and George (unpublished data) found streptococci in 35 of the 110 cases investigated by them, micrococci in 29 cases, *E. coli* in ten,

Paracolobactrum sp. in six, *Bacillus* sp. in four, and *Corynebacterium* sp. in one. It should be pointed out that the majority of the patients had been treated with sulfonamides; consequently, the incidence of various organisms may not represent that which would have been found in untreated cases of gas gangrene. The frequent occurrence of more than one bacterial form (especially, clostridium plus clostridium, or clostridium plus coccus) is a common characteristic of these infections.

TABLE 4

Clostridia Isolated from War Wounds

	PER CENT OF CASES	
	MacLennan (51) (259 cases)	Rustigian and Cipriani (87) (26 cases)
C. perfringens	23	27
C. novyi	5	8
C. septicum	2	8
C. tetani	6	0
C. butyricum	34	0
C. tertium	47	23
C. sporogenes	20	85
C. cochlearium	5	0
C. sphenoides	5	4
C. capitovale	4	0
C. tetanomorphum	3	0
C. bifermentans	0.7	4
C. hastiforme	0.3	0
C. putrificum		31
Unidentified strains	13	35

THE GENESIS OF THE INFECTION

It is apparent from the relatively high incidence of pathogenic clostridia in wounds which do not develop gas gangrene that factors other than the presence of the organisms are necessary before progressive infection can develop. Probably the most important factor is the local oxidation-reduction potential of the tissues in the immediate neighborhood of the organisms. That the oxidation-reduction potential of normal tissues may be too high for the growth of clostridia is indicated by the work of Hanke and Bailey (32), who found that *C. perfringens* (two strains) required an E_h lower than +74 millivolts at pH 7.4, *C. sporogenes* (1 strain) required an E_h lower than +114 mv. at pH 7.0, while *C. histolyticum* (1 strain) required an E_h lower than +76 mv. at pH 7.0. Hanke and Tuta (33) found the E_h of the circulating blood to vary between +126 and +246 mv., with 60% of the findings falling between +156 and +186 mv. From this information alone, one would not expect most strains of *C. perfringens* to be able to invade living tissue from the surface of a clean cut in which the adjacent tissue is in equilibrium with the circulating blood.

That gunshot wounds cannot be considered in this light is evident from the findings of workers who have investigated wounds and the mechanism of their formation by high-speed missiles. When a small missile strikes a heavily muscled portion of the body such as the thigh, it makes a small hole in the skin, a larger hole in the subcutaneous fat and then pierces the inelastic deep fascia surrounding the muscle through a hole just large enough to admit the missile. It then enters the muscle which responds by active reflex contraction, pulling the damaged fibers widely apart with the formation of a relatively large cavity (65). During this period, much of the kinetic energy of the missile is transformed into shock wave pressures. Regions of very high pressure are formed immediately in front and to each side of the moving missile. Relatively slow low pressure changes are connected with the behavior of the explosive temporary cavity formed behind the missile (34). The primary damage to the tissue is due to the crushing of the tissue in front of the missile and the stretching or tearing around the missile path which results from the formation and collapse of the temporary cavity (35). Localized and sharply defined areas of anoxic muscle are produced, probably as the result of local vascular spasm (44), and regions of extravasated blood are formed (35). Furthermore, since several muscles in the limbs are supplied by only one or two blood vessels (65), any damage to these results in larger areas of anoxic muscle. In this regard, MacLennan (51) pointed out that about 65% of the cases of gas gangrene he investigated were associated with damage to major blood vessels.

If muscle becomes anoxic, the oxidation-reduction potential will soon drop, for the phosphorolytic breakdown of carbohydrate will continue. Since the oxyhemoglobin and oxymyoglobin will soon be deoxygenated and no longer able to function as sources of oxygen, the anaerobic reduction of pyruvate to lactate will take place with a consequent drop in oxidation-reduction potential. Even if no muscle were involved, but only extravasated blood, the E_h would soon drop from about +170 mv. to about +50 mv. (97). The drop in potential in extravasated blood is also connected with phosphorolysis, for it does not take place in the presence of fluoride or iodoacetate.

The influence of atmospheric oxygen on the oxidation-reduction potential probably is not appreciable, for the rate of diffusion of oxygen through tissue is extremely low and the ability of muscle to consume oxygen is comparatively high; therefore one would not expect to find pO_2 of 0.01 atmosphere in mammalian muscle with non-functional blood supply beyond 0.1 to 0.2 mm from the surface (39).

As the oxidation-reduction potential of the anoxic muscle drops, the pH also is lowered because of the accumulation of organic acids. The relation between time and the drop in pH of muscle with impaired blood supply is shown by the results of Stoner and Green (96) in table 5. As a result of these changes in oxidation-reduction potential and pH, the catheptic enzymes of the muscle become active, hydrolyzing some of the protein and thus increasing the concentration of amino acids and peptides. In five hours, the concentration of the free amino acids would more than double, if the other amino acids increase in proportion

to tyrosine (96). After a longer time, the rate of hydrolysis probably becomes more rapid as the pH and oxidation-reduction potential of the muscle approach the optimal values for the action of the catheptic enzymes.

In addition to increasing the concentration of available amino acids, the increase in hydrogen ion concentration favors the growth of the clostridia in another way. Hanke and Bailey (32) found that there was a definite relation between pH and the highest oxidation-reduction potential at which various clostridia could grow. Their findings for *C. perfringens* are illustrated in table 6. If these findings are considered in connection with those of Stoner and Green presented in table 5, it appears that clostridia in the presence of anoxic muscle

TABLE 5

Decrease in pH in Anoxic Muscle

(From Stoner and Green (96))

Hours	0	1	2	3	4
pH of muscle	7.5	7.2	6.8	6.8	6.7

TABLE 6

Limiting E_h Values for Growth of C. perfringens

(From Hanke and Bailey (32))

pH	E_h (mv)
6.0	106
6.2	131
6.4	160
6.6	150
6.8	114
7.0	90
7.2	80
7.4	35–70
7.8	0–30

become much less demanding so far as the oxidation-reduction potential is concerned. A comparatively small drop in E_h brought about by glycolysis within the muscle should be sufficient to enable growth of the clostridia to start, if the pH of the muscle were around 6.5.

Probably the results of this interplay of factors have led the surgeon to shorten as much as possible the time between wound infliction and surgical intervention, i.e., the length of time within which anoxic muscle possibly contaminated with clostridia remains with a wound. The influence of the length of this period on the incidence of gas gangrene is indicated by the data in table 7, which is taken from the report of Langley and Winkelstein (43).

From the nutritional standpoint, also, it seems likely that some autolysis of tissue does take place before certain of the pathogenic clostridia can grow in the animal body. The amino acids found by Boyd, Logan, and Tytell (13, 14) to be

required by *C. perfringens* included cystine and tryptophan. No appreciable amount of cystine or cysteine exists as such in the intercellular fluids of the various organs, or in blood serum (98, 103). Glutathione, a compound which might serve as a convenient source of cysteine, is also absent from blood serum (103). Dent and Schilling (21) found no tryptophan in the blood plasma even when the specimens were taken shortly after the feeding of large amounts of protein. If the tissue were undergoing autolysis, however, these two amino acids would be supplied by the action of the catheptic enzymes mentioned above.

It seems likely, then, that there are two important factors necessary for the initiation of the growth of clostridia in muscle—an appreciable amount of tissue with impaired blood supply and a sufficient time lapse after wound infliction. Under such conditions, the action of the enzymes of the tissue probably provide

TABLE 7

Incidence of Gas Gangrene Related to the Interval Between Wound Infliction and Medical Attention

(*From Langley and Winkelstein (43)*)

	INTERVAL BETWEEN WOUNDING AND DRESSING	INTERVAL BETWEEN WOUNDING AND SURGERY	INCIDENCE PER 1000
American casualties	12 min	1.0 day	8
Free French casualties	18 min	1.5 day	12.3
Prisoners of war	340 min	3.75 day	51.9

a suitable hydrogen ion concentration, a suitable oxidation-reduction potential and sufficient amino acids or peptides to enable growth of the clostridia to start.

PROGRESS OF THE INFECTION

Once growth has started in anoxic muscle, progress of the invading organisms into undamaged muscle is dependent largely upon the production of exocellular enzymes and substances which interfere with the enzyme systems of the host's cells.

Of the pathogenic clostridia, only *C. perfringens* has been studied in detail, so far as the production of exocellular enzymes is concerned. Such investigations were greatly stimulated by the independent discovery by Nagler (66) and Seiffert (90) that the lecithin-splitting activity of culture filtrates could be correlated with the toxic activity. Further work demonstrated the identity of the lecithin-splitting enzyme and the alpha toxin, the main lethal component of culture filtrates (48, 50). Crook (19) concluded that there may be several lipolytic enzymes produced by *C. perfringens*. MacFarlane (47) found that sphingomyelin was attacked as well as lecithin, although cephalin (phosphatidylethanolamine or phosphatidylserine) was not attacked. Zamecnik *et al.* (104) reported that neither glycerophosphorylcholine nor lysolecithin was attacked. That *C. perfringens* produced one or more enzymes which could hydrolyze hyaluronic acid,

the "ground substance" or "intercellular cement" of the tissue, was shown by McClean (57). (For a review, see Meyer, 61.) A collagen-splitting enzyme (kappa toxin) was demonstrated by MacFarlane and MacLennan (49) and was further studied by Oakley, Warrack and van Heynigen (71), Evans (25), Bidwell and van Heynigen (12) and Bidwell (11). *C. perfringens* was found to produce also enzymes capable of hydrolyzing glycogen or starch (30, 93) and ribonucleic acid (93). Besides the proteolytic enzymes capable of hydrolyzing collagen, another is produced which hydrolyzes gelatin (55) and possibly other hydrolytic products of collagen. There is histological evidence only (83) for the production of an enzyme capable of depolymerizing desoxyribonucleic acid. Schiff (89) has reported that an exocellular enzyme that decomposed blood group A specific substance was found in culture filtrates of this organism.

The "bursting" factor, found in non-toxic filtrates by Fredette and Frappier (28) may be one or more of these enzymes, but until its properties are more clearly defined, it must be considered simply as an "aggressin."

Toxin and enzyme production of the other species of pathogenic clostridia have not been studied in such detail. However, Oakley, Warrack and Clarke (70) investigated the activity of filtrates of *C. novyi* with regard to toxic antigenic components, of which they found six. They designated the main lethal component the alpha toxin. The beta and gamma toxins were found to be hemolytic lecithinases. The delta and zeta toxins were also hemolytic, but were without lipolytic activity. The epsilon component was found to be responsible for the "pearly layer" phenomenon on nutrient agar. These workers found that the three types of *C. novyi* could be distinguished by the toxins formed; Type A formed alpha, gamma, delta, and epsilon; Type B formed alpha, beta and zeta; Type C regularly formed none of these, but irregularly formed gamma toxin.

Bernheimer (8, 9, 10) studied the lethal toxin of *C. septicum* which he considered to be identical with the hemolysin. He based the identity of the lethal toxin and the hemolysin on the following facts. 1. The lethal activity of various filtrates was proportional to the hemolytic activity. 2. Treatment with hydrogen peroxide reduced both proportionally. 3. They were adsorbed to the same extent by charcoal. 4. They were adsorbed to the same extent by kaolin. 5. Adsorption on erythrocytes removed lethal as well as hemolytic activity. 6. Both were destroyed in dilute solution at 36 C. 7. They were inseparable by fractional precipitation with ammonium sulfate. 8. Antihemolytic capacity of antitoxic horse sera was directly proportional to the antilethal capacity. In a study of the kinetics of hemolysis, he found that this resembled in several respects that of enzyme-catalyzed reactions, but differed in the absence of a clearly defined pH optimum. Bernheimer found also that *C. septicum* toxin was inactivated by the cooked meat present in some of the media he used. Preliminary extraction of the cooked meat with lipoid solvents prevented the inactivation. The lipids isolated in this way did not inactivate the toxin, however.

Detailed studies have been made of the action of crude *C. perfringens* culture filtrates on tissue by Robb-Smith (83) and by Frazer, *et al.* (27). The former investigator concluded that the histological changes could be ascribed to the

enzymes present in the culture filtrate, particularly lecithinase, collagenase, and hyaluronidase. All the histological changes found in muscle in gas gangrene due to *C. perfringens* could be reproduced *in vitro* with culture filtrates, except for edema and gas formation. Frazer, *et al.* also used culture filtrates *in vitro* and noted that visible breakdown of connective tissue occurred before any effect on muscle was noted. Tendon and nerve were more resistant than other tissues; on longer exposure, they too were affected. Dilatation and thrombosis of the microscopic blood vessels were regularly seen, as was swelling of the vascular endothelium. Kropp and Smith (42) investigated the tissue changes occurring in infections due to *C. perfringens* in guinea pigs, restricting their observations to the area immediately around the infection. They also emphasized the effect found on connective tissue, and the swelling and fragmentation of the collagenous fibers. Leukocytes, fibrocytes, reticular cells and histiocytes broke down rapidly, not only in the immediate vicinity of the invading organisms, but some little distance from them. Blood vessels were found to be thrombosed with the endothelium frequently ruptured.

The roles played by the individual enzymes in infections by *C. perfringens* have not been ascertained with certainty; such knowledge awaits the testing of the isolated enzymes in the animal body. However, it seems likely that the principal function of the enzymes in promoting the growth of *C. perfringens in vivo* may be tentatively outlined.

The glycogen-splitting enzyme probably serves to supply the organisms with carbohydrate for the energy for protoplasm and enzyme synthesis. The ribonuclease, and perhaps the desoxyribonuclease, aid indirectly in furnishing the organisms with adenine and with uracil, two factors necessary for optimum growth (13). The hyaluronidase, by breakdown of hyaluronic acid probably renders the tissues more permeable to the spread of the organisms and also furnishes them with N-acetyl-glucosamine, which stimulates the production of alpha toxin by some strains (85). The lecithinase serves to disrupt the cell membranes of various cellular elements of the tissues, including the phagocytic cells, and makes accessible to the other enzymes substrates which otherwise would be protected by the cell membrane. Furthermore, the lecithinase, as a result of its action on protein-lipid complexes in the muscle, frees appreciable amounts of various lipids (27, 49, 83), some of which serve to stimulate the formation of more lecithinase, as purified lecithin has been found to do (1).

The collagenase appears to be of prime importance in the progress of *C. perfringens* into uninfected muscle. Not only does this enzyme aid in breaking down the architecture of the muscle groups through destruction of connective tissue, but it also supplies the organisms with amino acids and peptides at a rate higher than that of the catheptic enzymes of the muscle. It probably also furnishes the factor necessary for lecithinase production which Adams, Hendee, and Pappenheimer (1) found in enzyme-hydrolyzed casein. Whether or not this factor is identical with that found by van Heynigen (38) in autolyzing horse muscle is uncertain. It is apparent, however, that the collagenase furnishes all the peptide-like substances required for growth and enzyme production by *C. perfringens*.

It appears that both the collagenase and lecithinase aid in the destruction of capillaries. The collagenous reticular sheath around the capillaries (56) is probably attacked by collagenase, while the endothelial cells composing the capillary walls apparently are destroyed by the lecithinase, resulting in increased capillary permeability. It appears that increase in capillary permeability (63) and the vasodilatation and thrombosis regularly seen (27, 42) cause the accumulation of fluid in intercellular spaces. As Mullally (65) has pointed out, such accumulation of fluid within the inelastic fascial sheath results in increased pressure which interferes with venous return and lymphatic drainage. The production of carbon dioxide and hydrogen from the fermentation of muscle glycogen and amino acids within the inelastic sheath further serves to accentuate pressure changes. With impairment of venous return and thrombosis of the capillaries, the blood supply to the immediate area is lessened, and with the consequent drop in oxidation-reduction potential and pH, a new portion of muscle is then made suitable for the growth of the clostridia, and the progress of the infection can continue.

It must be noted, however, in any consideration of the action of the exocellular enzymes, that Evans (22) was unable to find any evidence that antisera with considerable capacity for neutralizing hyaluronidase had any effect on the progress of infections by *C. perfringens*. This same worker (23) from the results of a study of lecithinase, hyaluronidase and theta toxin production of a number of strains of *C. perfringens*, concluded that virulence was generally associated with lecithinase—the production of hyaluronidase and theta toxin *in vitro* was not associated with virulence *in vivo*. With regard to sera with anti-collagenase activity (24), it was not demonstrated that it conferred any protection nor did it appear to enhance the protective properties of alpha antitoxin. However, in comparing different strains of *C. perfringens*, Evans (25) did find some relationship between the production of collagenase, the production of lecithinase and virulence for guinea pigs. It was pointed out that among the pathogenic clostridia, muscle destruction *in vivo* is produced to a marked degree only by the two collagenase-producing species, *C. perfringens* and *C. histolyticum*, and not by species incapable of producing collagenase (*C. tetani*, *C. novyi*, *C. septicum*).

A number of workers have investigated the relation between pathogenicity and *in vitro* production of hyaluronidase and lecithinase. The results of such experiments should be interpreted with caution where no relation is found to exist, unless the adequacy of the medium for toxin production by various strains has been considered. Growth of *C. perfringens* does not necessarily result in the production of exocellular enzymes, and there appears to be some variation from strain to strain so far as optimum conditions necessary for the production of the enzymes are concerned. Robertson and Keppie (84) compared the *in vitro* production of toxin of strains apparently responsible for active infections with strains which seemed to be present as wound contaminants. In general, strains from active infections were more toxigenic than the wound contaminants. McClean, Rogers and Williams (58) found that of 32 strains of *C. perfringens* studied by them 12 produced hyaluronidase, 11 of these being toxigenic. Of 20 strains that did not produce hyaluronidase, 11 were toxigenic. All of 20 strains of *C. septicum*

and 7 of 15 strains of *C. novyi* produced hyaluronidase. Keppie and Robertson (41) investigated strains of *C. perfringens* from various sources for pathogenicity in guinea pigs, production of alpha toxin, production of hyaluronidase, and formation of capsules. They found little relation between pathogenicity and the other characteristics. Kass, Lichstein and Waisbrenn (40) investigated the production of hyaluronidase and lecithinase in relation to virulence in 94 strains of *C. perfringens* isolated from soil and feces. They found 41 strains to be virulent for mice, of which 83% produced lecithinase and 47% hyaluronidase.

Altemeier and Furste (5) studied the virulence of 15 strains of *C. perfringens* isolated from soil and 25 strains isolated from wound infections of various degrees of severity. They found that the virulence of strains isolated from infected wounds was much greater than that of strains isolated from soil; that the virulence of strains isolated from localized infections was sometimes as high as the virulence of strains from fulminating cases; that there was no measurable correlation between the virulence of these strains for animals with closed wounds containing crushed muscle and dirt and their relative ability to produce alpha toxin, theta toxin, and hyaluronidase *in vitro;* and that the virulence as determined by the crushed muscle and dirt technique did not correspond with that determined by the simple injection of bacteria. It appears from these findings that factors other than exocellular enzyme production are of importance in determining virulence. Whether these factors are those stressed by Butler (16, 17)—capsule production, smooth colonies, and resistance to phagocytosis—cannot be determined from the evidence at hand. However, Miles (62) stated that there was a lack of association between the capacity of toxin-free suspensions to resist bactericidal properties of blood and the capacity to produce gas gangrene.

MacFarlane and MacLennan (49, 53) advanced the opinion that the lethal action of the alpha toxin on the host is not due to the enzyme activity *per se,* but is due to the absorption by the host of some toxic substance derived from muscle tissue which has been acted on by the lecithinase. Although no crucial evidence has been advanced, the following facts support this hypothesis: 1. Antitoxin containing antibody to alpha toxin is usually of slight value so long as infected muscle remains within the body. 2. A lethal dose of toxin given intramuscularly is larger than one given intravenously. 3. Alpha toxin given intravenously causes hemolysis of the red cells in the circulating blood, that given intramuscularly does not. 4. Toxin production *in vivo* seems to be associated with the growth of the clostridia in muscle. Growth of *C. perfringens* in other tissues usually does not yield infections similar to gas gangrene (51, 75). 5. Alpha toxin is rapidly and strongly adsorbed by muscle *in vivo.*

The interesting investigation of Zamecnik, Folch and Brewster (105) on the protection of animals against *C. perfringens* toxin was based on the assumption that protection was required against the lecithinase as such. They found that intravenous injection of certain purified lipids simultaneously with the toxin conferred temporary protection. Since only lipid preparations hydrolyzable by *C. perfringens* filtrates exerted such protective effect, it appeared that protection was afforded simply by supplying such an excess of substrate in the circulating

blood that only a comparatively small amount of lecithinase could attach to substrate in the tissues.

In another investigation, Zamecnik and Lipmann (106) found that antitoxin and lecithin competed for the lecithinase, providing an explanation for the coexistence in the circulating blood of both antitoxin and lecithinase. Since the enzyme-substrate complex was easily dissociable and the enzyme-antitoxin complex was much less so, eventually the antibody competed successfully for the enzyme. The physiological action of *C. perfringens* toxin and *C. novyi* toxin was also studied by this group (8, 144).

It seems unlikely from the work of Brown, McIntosh and White (17) that the histidine decarboxylase of *C. perfringens* has much effect on the host. Although histamine is formed by the decarboxylation of histidine, the increase in histamine content of gangrenous muscle (30 to 230%) seemed insufficient to cause appreciable effect, even locally.

CHEMOTHERAPY OF GAS GANGRENE

In spite of the advances in chemotherapy that have been made in recent years, it appears that none of the newer chemotherapeutic agents markedly affects the progress of clostridial infections of man. The sulfonamides have not shown any appreciable effect in the treatment of gas gangrene (18, 43, 51) and from the work of Ryan *et al.* (88) it appears that the use of analogs of growth factors other than *p*-aminobenzoic acid offer but little hope. Antibiotics other than penicillin tested by these workers—penicillin B (notatin), gliotoxin, gramicidin, tyrothricin, streptothricin and streptomycin—were less effective in laboratory animals than was penicillin. Zinc peroxide, urea peroxide, and pyrophosphate peroxide were also ineffective.

Although some clostridial infections in laboratory animals can be controlled by the use of penicillin, the effect of this agent in cases of gas gangrene is slight. MacFarlane (46) concluded that it could not be demonstrated that penicillin decreased the mortality rate in 185 cases considered by her (see table 8). It should be pointed out, however, that there is a possibility that the doses of penicillin used in humans were too low. Altemeier, Furste, and Culbertson (6) concluded, from the results of a well-planned series of experiments in guinea pigs, that human dosage of penicillin in cases of gas gangrene should be about 8 million units a day. The dosage employed by most surgeons in the armed forces was 200,000 to 400,000 units per day (99). Also, it is possible that penicillin is rendered inactive by reducing substances produced by the disintegrating tissue and the growing bacteria. There is evidence suggesting that penicillin acts by promoting the dehydrogenation of —SH groups to S—S groups more rapidly than the organisms can restore —SH groups (78). Most pathogenic clostridia produce hydrogen as one endproduct of carbohydrate and amino acid fermentation and their ability to restore —SH groups should be considerable. There is the further possibility that penicillin is inactivated locally by nucleic acid released from the cells of the host by the action of the bacterial or tissue enzymes, for Pandalai and George (73) have demonstrated that penicillin could be inactivated *in vitro* by nucleic acid.

The inability of the chemotherapeutic agents to prevent the initiation of clostridial infections is explicable if we consider that the bacteria grow first in muscle which is not in free equilibrium with the circulating blood. The agent can reach the organisms only by diffusion and this would be insufficient to allow the development of an appreciable concentration in a short time, since the concentration of a diffusing substance at any point will be inversely proportional to the square of the distance through which it has diffused. In considering the treatment of established infections with chemotherapeutic agents, it appears that here, also, there may be considerable difficulty in bringing the agent into contact with the organisms. As Miles and Miles (63) have shown, *C. perfringens*

TABLE 8

Penicillin and Mortality Rate

(From McFarlane (46))

	PENICILLIN			NO PENICILLIN			DIFFERENCE ± S.E.
	Cases	Deaths	Rate	Cases	Deaths	Rate	
Gas Gangrene of Arm or Leg							
Group I	23	6	26.1	11	1	9.1	−17 ± 15
Group II	8	2	25	30	16	53.3	28 ± 20
Group III	1	1	100	11	10	90.9	−9 ± 29
Gas Gangrene of Thigh, Buttock or Shoulder							
Group I	13	5	38.5	11	6	54.5	16 ± 20
Group II	8	6	75	32	26	81.3	6 ± 16
Group III	6	6	100	31	31	100	

Group I—Treatment within 6 hours of diagnosis, including surgery and antitoxin.
Group II—Treatment later than 6 hours or less than 50,000 units of antitoxin.
Group III—No surgery, no antitoxin.

toxin rapidly damages the capillaries, bringing about the exudation of fluid, first into the muscle itself and then into the loose tissue around the muscle. As pressure increases, leakage of fluid will cease and any agent given subsequently will get into the exudate only slowly, if at all, even if the circulation were otherwise unimpaired.

NOTES ON THE ISOLATION AND IDENTIFICATION OF CLOSTRIDIA FROM INFECTED WOUNDS

The isolation and identification of clostridia is somewhat more difficult than is the isolation and identification of many aerobic bacteria. Isolation is sometimes rendered tedious by nutritional requirements and by tendency to swarm. Identification is often troublesome because of discrepancies in reported characteristics and because of the apparently inherent variability of some strains.

With regard to complex nutritional requirements, some strains of the pathogenic clostridia are extremely fastidious when first isolated, growing very poorly on the surface of blood agar plates, even though the same medium will support

good growth of stock cultures of the same species. In general, the nutritional inadequacy of plating media is shown by the appearance on plates streaked from infected muscle of small satellite colonies of one species close to colonies of another. A basal medium prepared from infusion of fresh muscle or liver, or the addition of yeast extract thereto, usually supplies sufficient growth factors to satisfy even the fastidious strains.

Swarming may be lessened by the use of plating media with a relatively dry surface, as Reed (79) has suggested. A more nearly certain method is the use of plating media containing 4 to 6% agar. Colony form and hemolytic pattern on such media are not identical with those on media containing 1.5 to 2% agar, but the reduction in the swarming is well worth the extra labor involved in picking colonies of somewhat uncertain characteristics.

The biochemical characteristics of the clostridia most apt to be encountered in wounds have been described by Spray (94), Reed and Orr (80), and more recently by Reed (79). Since a number of species of clostridia are not considered in these reports, Bergey's Manual of Determinative Bacteriology should be consulted. The information offered by Weinberg *et al.* (102), is also of value.[2]

There are some discrepancies in the literature with regard to the characteristics of certain species. In general, the discrepancies concern the production of hemolytic zones on blood agar plates, the reduction of nitrate, the formation of indole and hydrogen sulfide, and the fermentation of certain carbohydrates. With regard to hemolytic zones around surface colonies, this is clearly a function of the plating medium as well as of the species of animal from which the blood was taken. The variation that can be obtained in this regard is illustrated by a comparison of the reports of Reed and Orr (80) and Reed (79). The former workers reported that no hemolytic zones were found around colonies of *C. tertium*, *C. fallax*, *C. sphenoides*, *C. capitovale* and *C. cochlearium* on blood agar plates, while in a more recent article Reed (79) described colonies of these species as being surrounded by zones of hemolysis.

Reed (81) has pointed out that the accumulation of indole in cultures of certain clostridia is governed by the relative rates of two reactions—the rate at which indole is formed and the rate at which it is broken down—and, consequently, that a slight modification in the rate of either reaction would be sufficient to change the results of a test for indole. A somewhat similar situation seems to exist with regard to the formation of nitrite from nitrate. This may explain, in some measure, the discrepancies that exist regarding the formation of indole by *C. sphenoides*, *C. capitovale*, and *C. tetani*, and the reduction of nitrate exhibited by *C. sphenoides*.

Lack of agreement as to the production of hydrogen sulfide probably stems largely from the use of different media by different investigators. As Reed and Orr (80) have pointed out, almost all species of clostridia will produce at least a trace of hydrogen sulfide if grown on a medium rich in organic sulfur. Different media have been used by various investigators to determine this characteristic.

[2] See also, Prévot: Manuel de classification et détermination des bactéries anaérobies. Masson, Paris, 1948.

Consequently, if the findings of one author are to be used for identification, the particular medium that he worked with must be used.

The lack of agreement with regard to the fermentation of various sugars by certain species can be explained only by the supposition that certain species are extremely variable. Richard (82) encountered variation within single strains in fermentation of sugars, except for glucose and fructose, and in the ability to hydrolyze starch upon repeated examination of strains of butyric acid bacteria. Likewise, Weinberg and Mihailesco (101) found a good deal of variation in the ability of *C. feseri* and *C. septicum* to ferment sucrose and salicin. Possible variability in the media as a cause of the lack of agreement has not been adequately explored.

In general, however, these discrepancies give rise to less difficulty than might be supposed, for other characteristics can often be used for tentative identification. The latter is particularly true of the toxigenic species, in which neutralization of toxin by monovalent antitoxin can be used. Also, the morphological characteristics seem to be comparatively stable, so that the determination of spore shape and position can usually be relied on to give worthwhile data. However, some species do not readily form spores. The determination of motility by the use of semi-solid agar also provides reliable information. However, since the great majority of the clostridia are motile, this information is of most value in the identification of the non-motile species. The proteolytic activity of various strains of the same species is usually quite uniform, and the determination of this characteristic provides data almost as reliable as does the determination of morphological characteristics. At the present time, there has not been sufficient work done on the antigenic composition of the clostridia to allow the use of serological methods, other than antitoxin neutralization, for the determination of identity.

It is apparent that our knowledge of the clostridia is still fragmentary. As the situation now stands, a number of species are ill-defined, and with regard to others, there is no agreement on the variation that is to be expected within a single species. It is for these reasons that workers in the field report rather large numbers of strains as "unidentified". There seems to be little hope that the situation will be clarified until detailed investigations, using modern methods, are made of the morphological and metabolic characteristics of many species. Such investigations should include the examination of a number of recently isolated strains, for there is some doubt that stock cultures always remain representative of their species.

A number of plating media have recently been devised for the tentative identification of certain species particularly the pathogenic ones. McClung and coworkers (59, 60) described an egg-yolk plating medium which they stated could be used for the presumptive identification of *C. perfringens*, *C. novyi*, *C. bifermentans*, *C. hemolyticum*, *C. botulinum*, *C. parabotulinum*, and *C. sporogenes*. Lyons and Owens (45) have recommended the use of Wilson-Blair medium for the rapid recognition of the clostridia involved in gas gangrene. They found that this medium was blackened by *C. perfringens*, *C. multifermentans*, *C. tertium*,

C. novyi, *C. sphenoides*, *C. septicum*, *C. bifermentans*, and *C. sporogenes*. Nagler (67) has described a medium containing 10% sheep red cells and 5% egg-yolk for the presumptive identification of *C. novyi*. Gordon and McLeod (31) have reported that heated-blood agar containing benzidine could be used for the rapid identification of this organism. Petrie (74) has suggested that specific antitoxin be added to plating media for the rapid identification of toxigenic species. Colonies of species against which the antitoxins were prepared were surrounded by concentric rings of precipitate. Hayward (36) has recommended the use of 20% human serum in plating media for the demonstration of *C. perfringens* by the Nagler reaction, increasing the specificity of the test by spreading half the plate with *C. perfringens* antitoxin.

The acrolein test for *C. perfringens* has been modified and advanced as a method to be used with mixed cultures (37). In this modification, the specimen is inoculated into glycerin broth which is incubated, and the presence of acrolein is demonstrated by the use of Schiff's reagent. Since this reagent is not specific for acrolein but will respond to almost any aldehyde, the specificity of the test for *C. perfringens* is doubtful. Similarly, the use of the "stormy fermentation" of milk for the demonstration of the presence of *C. perfringens* in mixed cultures has again been advanced (4), apparently without considering that other organisms which may give a similar reaction are also found in wounds. Furthermore, the use of a drop of the whey remaining after such a fermentation for the purpose of demonstrating lack of motility is not to be recommended.

In connection with such tests, it should be emphasized that the results are to be interpreted with caution. Because of the high incidence of pathogenic clostridia in wounds which never develop gas gangrene, the presence of a member of a pathogenic species cannot be taken as evidence that progressive infection has occurred, or even that it is likely to occur. Furthermore, in cases of active infection, the fact that a strain of a pathogenic species has been isolated does not necessarily indicate that it alone is responsible for the infection. Smith and George (91) pointed out that the distribution of clostridia in infected muscle is not always uniform. Consequently, the demonstration of one pathogenic species should not lead to the conclusion that it is the only one involved. Consideration must also be given to the possible roles played by the "non-pathogenic" clostridia and by the organisms not belonging to the genus. Quantitative data with regard to the numbers of various species present in a specimen would be of help and would also furnish desirable information regarding the role played by comparatively non-toxic strains, such as *C. sporogenes* or most strains of *C. bifermentans*, when they grow in association with toxigenic species.

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